DNA Damage-dependent Regulation and Function of \textit{akt-1} in \textit{Caenorhabditis elegans}

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Molecular Genetics
University of Toronto

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Department of Molecular Genetics
University of Toronto
2011

Abstract
The roundworm Caenorhabditis elegans possesses a single, conserved phosphatidylinositol 3-kinase (PI3K) signaling pathway that regulates somatic developmental decisions and lifespan through the Insulin-like receptor tyrosine kinase (RTK) DAF-2, the class I PI3K AGE-1 and the 3-phosphoinositide-dependent protein kinase 1 (PDK1) homologue PDK-1. This pathway ultimately controls the action of Akt homologues on the forkhead transcription factor DAF-16. The C. elegans Akt orthologue akt-1 also negatively regulates the DNA damage-dependent apoptosis of worm germ cells by indirectly interfering with activation of the key transcription factor CEP-1, the sole homologue of p53 in the worm. Because upstream regulation by RTK/PI3K signaling is known to couple with downstream Akt kinase activity, I hypothesized that the worm daf-2/age-1/pdk-1 pathway would function upstream of akt-1/Akt in response to DNA damage. Surprisingly, this was not the case: daf-2/InsR and pdk-1/PDK1 do not function upstream of akt-1/Akt and instead promote DNA damage-induced germ cell apoptosis independently of CEP-1/p53 by regulating the B cell lymphoma (Bcl2) homologue CED-9 and the Apoptotic protease-activating factor 1 (Apaf1)-like adapter protein CED-4, respectively. Furthermore, PDK-1/PDK1 promotes germ cell apoptosis by a mechanism that does not include changes in the subcellular localization or absolute levels of CED-4/Apaf1, but does require the
presence of CEP-1/p53. Therefore, daf-2/RTK, pdk-1/PDK1, and cep-1/p53 co-operate from independent pathways to drive germ cell death. The separation of worm Akt function from canonical RTK/PI3K regulation is consistent with the ability of AKT-1 to function without major changes in phosphorylation at threonine 350, a site homologous to Thr308 in mammals. Since this modification is an essential step in the activation of Akt proteins by PDK1, it is likely that damage-dependent germline activity of AKT-1 is controlled by a novel mechanism that does not involve phosphorylation by PDK-1 on key regulatory sites. These data argue that *C. elegans* rearranges single homologous components of a signalling pathway to respond to different stimuli *in vivo*. Finally, I present data identifying the *C. elegans* ataxia and telangetasia and Rad3-related (ATR) kinase homologue ATL-1 as a potential target of AKT-1. Collectively, my work has uncovered a novel DNA damage-dependent pathway that allows AKT-1 to control CEP-1/p53-dependent apoptosis.
Acknowledgments

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# Table of Contents

List of Tables ............................................................................................................................... viii
List of Figures ................................................................................................................................ ix
List of Abbreviations .................................................................................................................... xii

1 Introduction ............................................................................................................................... 1
   1.1 Programmed Cell Death ...................................................................................................... 1
   1.2 Types of Programmed Cell Death ..................................................................................... 1
   1.3 Using *Caenorhabditis elegans* to Study Programmed Cell Death ................................... 3
   1.4 *C. elegans* Germ Cell Apoptosis ...................................................................................... 5
      1.4.1 Physiological Germ Cell Apoptosis ............................................................................ 6
      1.4.2 DNA Damage-Induced Germ Cell Apoptosis ............................................................. 9
         1.4.2.1 DNA Damage ...................................................................................................... 9
         1.4.2.2 Signalling to Induce Apoptosis .......................................................................... 10
   1.5 The Phosphatidylinositol 3-kinase Pathway Opposes Apoptosis ...................................... 12
   1.6 Phosphatidylinositol 3-kinase Signalling in *C. elegans* .................................................... 17
      1.6.1 Developmental Arrest and Lifespan ........................................................................... 17
      1.6.2 Stress Resistance and Innate Immunity ..................................................................... 18
      1.6.3 DNA Damage-Induced Germ Cell Apoptosis ............................................................. 19

2 Materials and Methods ............................................................................................................ 22
   2.1 *Caenorhabditis elegans* Genetics .................................................................................... 22
   2.2 Quantification of Germ Cell Apoptosis ............................................................................. 22
   2.3 Statistical Analyses ......................................................................................................... 23
   2.4 Quantification of Germ Cell Number .............................................................................. 23
   2.5 Quantification of Apoptotic Cell Engulfment in *pdk-1(mg142)* ...................................... 24
   2.6 RNA Interference .......................................................................................................... 24
   2.7 Quantification of CEP-1/p53 Activity .............................................................................. 24
5.3 A Novel Regulatory Mechanism for Akt ................................................................. 77
5.4 ATL-1 as a Downstream Target of AKT-1 ................................................................. 83
5.5 Implications for Cancer ......................................................................................... 84
6 Future Directions ....................................................................................................... 86

6.1 Regulation of DNA Damage-induced Germ Cell Apoptosis by daf-2/InsR and pdk-1/PDK1 .............................................................. 86
6.2 Activation and Control of AKT-1/Akt in Response to DNA Damage ..................... 89
6.3 Are The Observed Inversions and Bifurcations in C. elegans Phosphatidylinositol 3-kinase Signalling Conserved in Mammals? ... 92
6.4 How Does AKT-1/Akt Regulate ATL-1/ATR to Dampen Pro-apoptotic Signalling? 95
References ..................................................................................................................... 96
List of Tables

Table 3.1 *pdk-1* does not regulate the clearance of apoptotic cells ........................................... 33

Table 3.2 *daf-2* and *pdk-1* have a minor role in germline proliferation........................................ 34

Table 3.3 Loss of *pdk-1* cannot rescue the sterility of *ced-9(0)* worms........................................ 41
List of Figures

Figure 1.1 Types of programmed cell death. ................................................................. 3

Figure 1.2 The C. elegans life cycle. ............................................................................. 4

Figure 1.3 Hermaphrodite germline anatomy............................................................. 6

Figure 1.4 Morphology of developmental and physiological germ cell apoptosis in C. elegans... 8

Figure 1.5 Radiation-induced DNA damage. ............................................................. 10

Figure 1.6 Pathway eliciting DNA damage-induced germ cell apoptosis.................... 11

Figure 1.7 Phosphoinositide chemistry....................................................................... 13

Figure 1.8 Phosphatidylinositol 3-kinase signalling in mammals. ............................ 15

Figure 1.9 The C. elegans PI3K pathway................................................................. 18

Figure 1.10 Regulation of damage-induced apoptosis by AKT proteins...................... 20

Figure 3.1 Structure of DAF-2/InsR and location of daf-2 mutations used in this study. .... 30

Figure 3.2 PI3K signalling components promote DNA damage-induced germ cell apoptosis. .. 32

Figure 3.3 daf-2 and pdk-1 do not function upstream of akt-1. ................................. 36

Figure 3.4 daf-2 and pdk-1 regulate the core apoptosis pathway independently of cep-1/p53 and akt-1. ............................................................................................................................................. 39

Figure 3.5 CED-4 localization does not change in response to IR or with respect to pdk-1 status. .................................................................................................................................................................................... 43

Figure 3.6 cep-1 and pdk-1 function in parallel to promote damage-induced germ cell apoptosis. .................................................................................................................................................................................... 45

Figure 3.7 akt-1 is not regulated by kinases known to function upstream of Akt in mammals. .. 47
Figure 3.8 Phosphorylation of AKT-1 at Thr350 and Ser517 does not change in response to IR. ............................................................................................................................................................ 50

Figure 3.9 daf-2 and pdk-1 differentially regulate Thr350 and Ser517 phosphorylation status... 53

Figure 3.10 akt-1 functions in the germline to control damage-induced germ cell apoptosis...... 57

Figure 4.1 ATL-1 contains putative Akt consensus phosphorylation sites, as detected by Scansite. .......................................................................................................................................................... 60

Figure 4.2 The gk186 allele of atm-1 does not cause resistance to IR-induced apoptosis. ....... 62

Figure 4.3 atm-1 does not function downstream of akt-1 in response to ultraviolet (UV) radiation. .......................................................................................................................................................... 62

Figure 4.4 Loss of atl-1 suppresses DNA damage-induced germ cell apoptosis in akt-1(RNAi) worms.......................................................................................................................................................... 64

Figure 4.5 akt-2 functions independently of atl-1 to regulate DNA damage-induced germ cell apoptosis. .......................................................................................................................................................... 65

Figure 4.6 Single-stranded DNA serves as a common substrate for ATR activation.............. 68

Figure 4.7 AKT-1 could target multiple aspects of ATL-1 regulation...................................... 70

Figure 4.8 Ser1159, a site of Akt phosphorylation in human TopBP1, may not be conserved in MUS-101................................................................................................................................................. 71

Figure 5.1 Phosphatidylinositol 3-kinase pathway components regulate DNA damage-induced germ cell apoptosis at multiple levels of the C. elegans apoptotic signalling pathway........... 76

Figure 5.2 Phosphorylation in the activation loop of kinases clears the active site for catalysis. 78

Figure 5.3 An activation loop binding partner-dependent mechanism for AKT-1 activation..... 81

Figure 5.4 Hypothetical functional output switching by stimulus-specific phosphorylation of AKT-1. .......................................................................................................................................................... 82

Figure 5.5 A model for the DNA damage-dependent regulation of AKT-1......................... 84
Figure 6.1 Loss of *daf-16* only partially reverts the resistance to apoptosis of *daf-2* mutants. .... 88

Figure 6.2 Stimulus- and output-specific selection of phosphatidylinositol 3-kinase signalling proteins may represent a conserved mechanism to generate functional diversity. ......................... 93
List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>9-1-1</td>
<td>RAD9-RAD1-HUS1 trimeric clamp complex</td>
</tr>
<tr>
<td>AGE</td>
<td>AGEing alteration</td>
</tr>
<tr>
<td>AO</td>
<td>acridine orange</td>
</tr>
<tr>
<td>APAF</td>
<td>Apoptotic Protease-Activating Factor</td>
</tr>
<tr>
<td>ATL</td>
<td>ATm-Like</td>
</tr>
<tr>
<td>ATM</td>
<td>ataxia and telangectasia-mutated</td>
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<td>ATR</td>
<td>ataxia and telangectasia and Rad3-related</td>
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<td>ATRIP</td>
<td>ATR-interacting protein</td>
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<td>Bcl2 antagonist of cell death</td>
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<td>Bcl</td>
<td>B Cell Lymphoma</td>
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<td>bHLH</td>
<td>basic helix-loop-helix</td>
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<td>BRCT</td>
<td>breast cancer susceptibility protein C-terminal domain</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<td>calcium and calmodulin-dependent protein kinase kinase</td>
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<tr>
<td>Caspase</td>
<td>cysteiny1 aspartate-specific protease</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin-dependent kinase</td>
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<td>CED</td>
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<td>CEP</td>
<td>C. Elegans P53-like protein</td>
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<td>CES</td>
<td>CEl1 death Specification</td>
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<td>CKK</td>
<td>Calcium-calmodulin Kinase Kinase</td>
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<td>CLocK (biological timing) abnormal</td>
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<tr>
<td>DAF</td>
<td>abnormal DAuer Formation</td>
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<td>4',6-diamidino-2-phenylindole</td>
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<td>Differential Interference Contrast</td>
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<td>disodium ethylenediaminetetraacetate</td>
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<td>epidermal growth factor receptor</td>
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<td>EGL</td>
<td>EGg Laying defective</td>
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<td>green fluorescent protein</td>
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<tr>
<td>GSK</td>
<td>glycogen synthase kinase</td>
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<tr>
<td>HPR</td>
<td>Homolog of s.Pombe Rad</td>
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<td>HRP</td>
<td>horseradish peroxidase</td>
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<tr>
<td>HUS</td>
<td>HydroxyUrea Sensitive</td>
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<tr>
<td>IPTG</td>
<td>isopropyl β-D-1-thiogalactopyranoside</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>IR</td>
<td>Ionizing radiation</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin receptor substrate homolog</td>
</tr>
<tr>
<td>IST</td>
<td>Insulin receptor Substrate homolog</td>
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<tr>
<td>KIN</td>
<td>Protein Kinase</td>
</tr>
<tr>
<td>KRI</td>
<td>Human KRIt1 (Krev interaction trapped/cerebral cavernous malformation 1) homolog</td>
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<td>L1-L4</td>
<td>Larval stages 1-4 in <em>C. elegans</em></td>
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</tr>
<tr>
<td>LET</td>
<td>LEThal</td>
</tr>
<tr>
<td>LIN</td>
<td>Abnormal cell Lineage</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>Mdm2</td>
<td>Murine double minute 2</td>
</tr>
<tr>
<td>MRN</td>
<td>MRE11(Meiotic REcombination)-RAD50-NBS1(Nijmegen Breakage Syndrome)</td>
</tr>
<tr>
<td>MRT</td>
<td>Mortal germline</td>
</tr>
<tr>
<td>MO25</td>
<td>Mouse embryo scaffolding protein 25</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>mTORC1</td>
<td>Mammalian target of rapamycin complex 1, defined by Raptor (rapamycin-sensitive companion of TOR)</td>
</tr>
<tr>
<td>mTORC2</td>
<td>Mammalian target of rapamycin complex 2, defined by Rictor (rapamycin-insensitive companion of TOR)</td>
</tr>
<tr>
<td>MUS</td>
<td>Mutagen Sensitive</td>
</tr>
<tr>
<td>NDS</td>
<td>Normal Donkey serum</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappa B</td>
</tr>
<tr>
<td>NGM</td>
<td>Nematode growth medium</td>
</tr>
<tr>
<td>NHEJ</td>
<td>Non-homologous end joining</td>
</tr>
<tr>
<td>NP</td>
<td>Nonyl phenoxypolyethoxylethanol</td>
</tr>
<tr>
<td>NSM</td>
<td>Neuro-secretory motor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCD</td>
<td>Programmed cell death</td>
</tr>
<tr>
<td>PDK1</td>
<td>3-phosphoinositide-dependent protein kinase 1</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin homology</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
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<tr>
<td>PI4K</td>
<td>Phospholipid-Independent Akt Kinase</td>
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<td>PIKK</td>
<td>Phosphatidylinositol 3-kinase-like kinase</td>
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<td>Phosphatidylinositol-4,5-bisphosphate</td>
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<tr>
<td>PKA</td>
<td>Protein kinase A</td>
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<tr>
<td>PMSF</td>
<td>Phenylmethanesulfonylfluoride</td>
</tr>
<tr>
<td>PPW</td>
<td>Paz (piwi/argonaute/zwille)/PiWi (p-element induced wimpy testis) domain-containing</td>
</tr>
<tr>
<td>PtdIns</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
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<td>-----------</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene fluoride</td>
</tr>
<tr>
<td>pY</td>
<td>phospho-tyrosine</td>
</tr>
<tr>
<td>RAD</td>
<td>RADiation sensitive</td>
</tr>
<tr>
<td>Ras</td>
<td>rat sarcoma</td>
</tr>
<tr>
<td>Rb</td>
<td>retinoblastoma</td>
</tr>
<tr>
<td>RICT</td>
<td>Rapamycin-Insensitive Companion of TOR homolog</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RPA</td>
<td>replication protein A</td>
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<tr>
<td>RRF</td>
<td>Rna-dependent Rna polymerase Family</td>
</tr>
<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<tr>
<td>SDS-PAGE</td>
<td>SDS-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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<tr>
<td>SGK</td>
<td>Serum and Glucocorticoid-inducible Kinase</td>
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<tr>
<td>SH2</td>
<td>Src homology domain 2</td>
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<tr>
<td>SIR</td>
<td>yeast SIRtuin related</td>
</tr>
<tr>
<td>SLI</td>
<td>Suppressor of LIneage defect</td>
</tr>
<tr>
<td>ssDNA</td>
<td>single-stranded DNA</td>
</tr>
<tr>
<td>STK11</td>
<td>serine/threonine kinase 11</td>
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<tr>
<td>STRAD</td>
<td>STE20-related adapter protein</td>
</tr>
<tr>
<td>TBG</td>
<td>TuBulin, Gamma</td>
</tr>
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<td>TBST</td>
<td>Tris-buffered saline-0.1% v/v Tween-20</td>
</tr>
<tr>
<td>Tel</td>
<td>TELomere maintenance</td>
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<tr>
<td>TopBP1</td>
<td>topoisomerase II binding protein 1</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
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1 Introduction

1.1 Programmed Cell Death

Organisms and their constituent cells regularly encounter noxious stimuli that can hinder their functioning. A key decision for each metazoan cell is whether to attempt to repair and survive in the face of this adversity, or to die for the benefit of the entire organism. This decision tree, while complex (discussed below) is initiated by cellular sensing mechanisms that detect potential damage and initiate downstream signaling processes. Furthermore, the decision between life and death is not limited to this context and is essential for the development of many multicellular organisms. For example, metamorphosis of the tadpole into the adult frog requires the regulated death of tail cells, while the interdigital epidermis in humans is removed by regulated death to facilitate the flexible digital movements that we take for granted. Indeed, programmed cell death is a process used in many developmental situations to cull excess cells, sculpt the final forms of tissues, and to ensure the survival of the entire organism (Jacobson et al., 1997). One major disease that can result from the dysregulation of programmed cell death is cancer. Achieving dysregulation of the cell death process, neoplastic cells can continue to proliferate in the face of significant intrinsic damage as well as exogenously provided toxins (i.e. chemotherapy), rendering them a significant threat to the health of the afflicted organism (Hanahan and Weinberg, 2000). Thus, increased understanding of the cell death process has direct implications for our understanding of many human diseases.

1.2 Types of Programmed Cell Death

Programmed Cell Death (PCD) can be subdivided into two main types, principally by morphologic criteria. Type I PCD is termed apoptosis and involves the orderly destruction of the cell first by separation from its tissue partners, followed by nuclear and cytoplasmic condensation, fragmentation of the condensed cell and finally, phagocytosis of the resultant fragments by neighbours or dedicated phagocytes (Kerr et al., 1972) (Figure 1.1). Apoptosis was originally noted for its wide-ranging role in a variety of developmental situations and its distinct appearance in cancerous tumours during both their growth and therapy-induced regression.
Indeed, this form of cell death is often the choice of cells that die after exposure to noxious stimuli such as DNA damage.

Type II PCD, known as autophagic death, involves the self-destruction of cells from the inside-out, hence the literal translation of “self-eating” (Bredesen et al., 2006). Autophagy is recognized by massive autophagocytosis of intrinsic cellular contents and is usually induced in response to starvation (Figure 1.1). This frees additional nutrients for the cell and can presumably facilitate survival in lean times. A causal role for autophagy in cell death has been debated, although recent studies have suggested that it does play a key role in the developmental death of salivary glands in *Drosophila* (Berry and Baehrecke, 2007).

In addition to PCD, there exist other types of cell death, all described by morphological criteria as well (Kroemer et al., 2009). Most important to this discussion, and to cell death in cancer specifically, the type of cell death termed necrosis involves nuclear and cytoplasmic swelling, followed by plasma membrane rupture and the spillage of cellular contents into the surrounding tissue (Bredesen et al., 2006) (Figure 1.1). Unlike apoptosis and autophagy, necrosis represents the untimely death of unhealthy cells. It can be detrimental to surrounding cells and this may be why it is not generally seen in physiologic contexts such as development.
Apoptosis, or Type I PCD, involves segregation of a dying cell from neighbours, nuclear-cytoplasmic condensation, followed by fragmentation of the cell and phagocytosis of these fragments by surviving neighbours. Autophagy (Type II PCD) is distinguished by excessive autophagocytosis; a causal role for this in cell death is still controversial. Both apoptosis and autophagy are self-contained methods of cell death; necrosis differs in that cell membrane rupture and spillage of cellular contents follows nuclear and cytoplasmic swelling.

1.3 Using *Caenorhabditis elegans* to Study Programmed Cell Death

*C. elegans* is a small, soil-dwelling nematode that undergoes a three day life cycle at 20°C. Eggs are laid by the hermaphroditic sex, using sperm generated autologously. Upon hatching, worms progress through four larval stages (1-4), molting between each stage until the adult form is reached (Figure 1.2). Egg-laying then begins the cycle again. This rapid life cycle, coupled with hermaphroditic reproduction was initially exploited in genetic studies aimed at understanding the structure of a simple nervous system *in vivo* (Brenner, 1974).
Figure 1.2 The *C. elegans* life cycle.

*C. elegans* eggs hatch to release L1-stage larvae that molt through three other larval stages to generate the adult form. If environmental stress is excessive during the L1 stage, larvae can enter into the alternative L3 stage known as Dauer [for “enduring” (Cassada and Russell, 1975)]. Return of favourable conditions induces exit from Dauer and the life cycle is resumed at the L4 stage. The non-Dauer life cycle of *C. elegans* occurs over ~3 days at 20°C. Dauer larvae can survive for at least six months under adverse conditions. Anterior is left on each worm.

Additionally, exhaustive lineage analysis demonstrated that of 1090 somatic cells generated, 131 die by apoptosis (Sulston, 1976; Sulston and Horvitz, 1977; Sulston et al., 1983). Since apoptosis can be morphologically distinguished microscopically (Figure 1.4A) and all *C. elegans* life stages are transparent to visible light, further studies were able to identify mutations that precluded these developmental apoptoses (Ellis and Horvitz, 1986; Horvitz et al., 1983). These studies demonstrated, for the first time, that the ability of a cell to undergo apoptosis was under genetic control and therefore, PCD was a regulated mechanism for cell death. Following upon these contributions, early studies defined the largely invariant apoptosis seen during development - now known as “developmental apoptosis”. Later studies found that developmental apoptosis is controlled by tissue specific transcriptional activation of the *egl-L* gene (*egl – EGg Laying defective*). For example, in the death of the neuro-secretory motor (NSM) neuron sister cells
(Figure 1.4C), the two basic Helix-Loop-Helix (bHLH) transcription factors HLH-2 and HLH-3 promote egl-1 expression (EGL-1 antagonizes CED-9 activity; see below). The cell-lineage-specific Slug transcription factor homologue CES-1 (Metzstein and Horvitz, 1999) (CES – CELL death Specification) antagonizes HLH-2 and HLH-3 at the egl-1 promoter (Thellmann et al., 2003). By expression of the Hepatic Leukemia Factor (HLF) transcription factor homologue CES-2 (Metzstein et al., 1996; Wu et al., 2005) in the NSM sisters, CES-1 expression is repressed, resulting in activation of egl-1 by HLH-2/HLH-3. This ensures the normal death of the NSM sister cells. Other cells that die by apoptosis during C. elegans development use similar a strategy to antagonize CED-9 by regulating egl-1 expression with cell lineage-specific transcription factors (Nehme and Conradt, 2008). Additional studies identified apoptosis in the germ line and found that it exhibits overlapping, but distinct genetic requirements to those of developmental apoptosis (Gumienny et al., 1999).

1.4 C. elegans Germ Cell Apoptosis

The C. elegans hermaphrodite body consists of a hypodermal tube within which are located body wall and pharyngeal muscles, a gut, a nervous system, and a germline, among other tissues. In fact, the germline occupies much of the volume of the body, being arranged as a bi-cornate structure whose two proximal ends are connected at a central uterus. Each germline arm extends away from the uterus, and at half of its total length, reflexes on itself to generate distinct regions: the proximal and distal gonad arms (Figure 1.3). The distal tip of each arm lies in close proximity to its symmetrically-related partner, both ultimately residing dorsal to the uterus. In the distal arm of each gonad exists a population of mitotically proliferating germ cells. These cells are maintained in their mitotic state by a variety of influences, such that the transition out of this state is spatially well demarcated in the distal arm (Kimble and Crittenden, 2005). Upon exit from the mitotic region, germ cells enter prophase I of meiosis and pass through leptotene, zygotene and pachytene as they progress down the distal arm. After the reflexed bend in the gonad, germ cells exit pachytene and thus enter the proximal arm in diplotene. Here, they cellularize and enlarge to form definitive gametes – up until this point, germ cells have existed as a syncytium. In the L4 stage, meiotic germ cells differentiate to form a limited number of sperm (~150 per gonad). Later, diplotene germ cells differentiate into diakinetic oocytes. Here, these
oocytes arrest meiotic proliferation until they are fertilized by autologous sperm (see above) in the spermatheca (Greenstein, 2005). While spermatogenesis in the hermaphrodite occurs only during the L4 stage, oogenesis continues for much of the adult lifespan (Hubbard and Greenstein, 2005). *C. elegans* hermaphrodites produce a limited number of self-fertilized progeny (~300). *C. elegans* males, on the other hand, produce only sperm throughout adulthood.

Figure 1.3 Hermaphrodite germline anatomy.

Mitotic germ cells (yellow) proliferate and enter early meiosis I (green). Germ cells pass through pachytene (red) of prophase I as the gonad reflexes upon itself. They then progress through diplotene and diakinesis (blue) as part of oogenesis, before being fertilized in the spermatheca (lavender). Fertilized eggs (orange) are extruded from the vulva (not shown). Direction of germ cell maturation (distal to proximal) is depicted with an arrow.

1.4.1 Physiological Germ Cell Apoptosis

Physiological apoptosis of maturing germ cells occurs in the reflexed bend of the gonad, coincident with the exit from pachytene (Gumienny et al., 1999) (Figure 1.4B). Interestingly, this type of apoptosis only occurs in hermaphrodites. Proper meiotic progression is required for physiological apoptosis to occur since mutations in the mitogen-activated protein kinase (MAPK) pathway that block pachytene exit (Church et al., 1995) also prevent apoptosis (Gumienny et al., 1999). Most importantly, however, physiological germ cell apoptosis is dependent on a conserved genetic pathway that utilizes the same components as those used by
developmental apoptosis (Figure 1.4C). Physiological germ cell apoptosis is absolutely dependent on the ced-3 and ced-4 genes (ced – CEll Death abnormal). These two loci encode, respectively, a Caspase (cysteiny1 aspartate-specific protease) (Yuan et al., 1993) and the worm homologue of the human Apaf1 (apoptotic protease-activating factor 1) adapter protein (Yuan and Horvitz, 1992; Zou et al., 1997). These two protein products directly interact to initiate the proteolytic activity of CED-3 and drive germ cell death (Chinnaiyan et al., 1997a; del Peso et al., 1998; Irmler et al., 1997; Seshagiri and Miller, 1997; Wu et al., 1997; Yang et al., 1998). CED-4 acts upstream of CED-3 (Shaham and Horvitz, 1996), promoting activation of the pro-enzyme (Qi et al., 2010). The CED-9 protein impedes this activation of CED-3 by directly interacting with CED-4 (Chinnaiyan et al., 1997b; James et al., 1997; Ottilie et al., 1997; Spector et al., 1997; Wu et al., 1997; Yan et al., 2005). ced-9 encodes the worm homologue of bcl2 (B cell lymphoma) (Hengartner and Horvitz, 1994), a potent anti-apoptotic protein in mammals. In C. elegans, ced-9 inhibits physiological germ cell as wells as developmental apoptosis. The physiological signal that instructs CED-9 to release CED-4 and thus allow CED-3 activation has not been identified.
Figure 1.4 Morphology of developmental and physiological germ cell apoptosis in *C. elegans*.

A) Developmental apoptosis occurs as part of an invariant program during *C. elegans* embryogenesis. Dying cells can be visualized directly (white arrow). The normal death of the NSM sister neurons is outlined in C. Anterior is at right. B) Germ cells dying physiologically in the pachytene region of the germ line are shown (white arrows). The pathway controlling this process is depicted in C. Distal is to the right at top; proximal, to the right at bottom. C) Pathways controlling developmental and physiological germ cell apoptosis. (Left) Death of the NSM sister cells during development is controlled by the expression of *egl-1* (Thellmann et al., 2003). An heterodimer of the basic Helix-Loop-Helix (bHLH) transcription factors HLH-2/HLH-3 binds to the *egl-1* promoter and activates its expression. The CES-1 transcription factor, homologous to Slug in mammals (Metzstein and Horvitz, 1999), competitively inhibits HLH-2/HLH-3 by binding to the same site in *egl-1* (Thellmann et al., 2003). CES-2,
homologous to the Hepatic Leukemia Factor (HLF) transcription factor (Metzstein et al., 1996; Wu et al., 2005), represses expression of ces-1 so that death of the NSM sisters can occur normally. (Right) Physiological germ cell death is promoted by CED-3 and CED-4. CED-9 antagonizes this process. Because EGL-1 does not control CED-9 in this context, the pathways that elicit physiological germ cell apoptosis await discovery (red question mark).

1.4.2 DNA Damage-Induced Germ Cell Apoptosis

1.4.2.1 DNA Damage

DNA damaging agents all attack the structure of DNA. These modifications can eventually lead to deleterious mutations that affect gene expression/function. While there are many different damaging agents, I will focus on the mechanism of action of ionizing radiation (IR), one of the most potent damaging agents encountered by cells (Figure 1.5). Ionizing radiation consists of high-energy particulate radiation, such as α-particles, or high-energy electromagnetic radiation such as X-rays or γ-rays; it leads to the generation of free radicals and ions in its wake (Ward, 2000). γ-rays, the principal damaging agent used in this study, encounter biological structures and dissipate their energy into the outermost electron orbitals. Often, sufficient energy is contained within a γ photon to cause the ejection of one or more of these outer electrons, simultaneously generating a residual radical and a free electron. These free electrons and radicals can be of sufficient energy to repeat this process when they encounter additional biomolecules (Ward, 2000). In the case of DNA, absorption of γ photons leads to two main outcomes: breaks in the strands of the double helix; and the generation of free radicals that can attack adjacent sites. It was originally thought that the double strand break was the principal lesion associated with lethality (Little, 2000), but additional work has demonstrated that ionizing radiation also leads to a complex mixture of radical-induced lesions that surround the break, contributing significantly to downstream mutations (Ward, 2000).

How mutations arise is best understood if one considers the mechanisms cells use to repair these lesions. Mammalian somatic cells generally favour the imprecise re-joining of double strand breaks via a process known as non-homologous end joining (NHEJ) (Reddy and Vasquez, 2005). NHEJ can work quite effectively with clean break ends, but simultaneous lesions affecting the ends of the breaks can decrease the precision of the process and contribute to re-joining that
either excludes or duplicates intervening sequences (Figure 1.5). In certain circumstances this can also result in the insertion of novel intervening sequences as well. In the soma, *C. elegans* generally uses NHEJ to repair IR-induced double strand breaks, the germline instead favours homologous recombination as a means to protect the integrity of the genome (Clejan et al., 2006). Repair by homologous recombination repair is generally of higher fidelity than NHEJ (Krogh and Symington, 2004), but can still lead to mutations through defects in the resolution of recombination intermediates or by repair in regions of highly repetitive sequence. Regardless of the ultimate mechanism used to effect repair, the presence of DNA damage is detected by an evolutionarily conserved pathway that determines the future viability of damaged cells.

![Figure 1.5 Radiation-induced DNA damage.](image)

Mutations in DNA can arise when non-homologous end joining is used to ligate double strand breaks that have incurred additional damage from radicals or that have been resected before repair.

### 1.4.2.2 Signalling to Induce Apoptosis

DNA damage can occur in a variety of forms, and recognition of these various types of damage requires a flexible yet sensitive system. In the *C. elegans* hermaphrodite germline, DNA damage elicited by IR is sensed by two genetically separable detectors (Figure 1.6).
Figure 1.6 Pathway eliciting DNA damage-induced germ cell apoptosis.

Mammalian homologues of *C. elegans* pathway components are listed in brackets. See text for details.

The first, encoded by the products of the *hpr-9*, *mrt-2* and *hus-1* genes (*hpr* – Homologue of *s. Pombe* Rad; *mrt* – MoRTal germline; *hus* – HydroxyUrea Sensitive) represents the worm homologue of the mammalian Rad9-Rad1-Hus1 DNA damage sensing complex (Ahmed and Hodgkin, 2000; Hofmann et al., 2002) (*Rad* – Radiation sensitive). The so-called “9-1-1” complex forms a heterotrimeric clamp that surrounds DNA (Dore et al., 2009) and, in concert with other factors, activates downstream pro-apoptotic signalling (Gartner et al., 2000). In mammals, 9-1-1 is recruited specifically to sites of single-stranded DNA flanked by the 5' end of a complementary primer, a common structure that is presumably generated from a variety of initial lesions (MacDougall et al., 2007; Majka et al., 2006b). Co-recruitment of the large phosphatidylinositol 3-kinase-like kinase (PIKK) Ataxia and Telangectasia and Rad3-related (ATR), termed ATL-1 in the worm (ATL – Ataxia and Telangectasia-Like), initiates downstream signaling in concert with lesser contributions from the Ataxia and Telangectasia-mutated (ATM) protein kinase homologue ATM-1 (Aoki et al., 2000; Garcia-Muse and Boulton, 2005; Stergiou et al., 2007). ATL-1 signals through the checkpoint kinase CHK-1 (Lee et al., 2010) to the p53 homologue CEP-1 (Derry et al., 2007; Schumacher et al., 2001) (*CEP* – *C. Elegans* P53-like protein), which serves as a central integrator of DNA damage-induced apoptosis signalling.

The second pathway that detects DNA damage in the germline (Figure 1.6) is defined solely by the *clk-2* gene (*clk* – CLocK (biological timing) abnormal), which encodes a homologue of the yeast Tel2 protein (Ahmed et al., 2001; Benard et al., 2001) (*Tel* – TELomerase maintenance). Interestingly, while Tel2 is key to the maintenance of telomeres in *Saccharomyces cerevisiae*, it is not required for this in *C. elegans* (Ahmed et al., 2001). Because *clk-2* functioned
independently of hus-1 and mrt-2, it was inferred that these genes define independent DNA damage sensing pathways (Ahmed et al., 2001; Hofmann et al., 2002). However, more recent studies in mammals and yeast have suggested that Tel2 proteins may function in the same pathway as the 9-1-1 complex to control activation of damage-responsive PIKKs (Anderson et al., 2008; Rendtlew Danielsen et al., 2009; Takai et al., 2007). The mechanism by which clk-2 senses damage and signals to cep-1 is currently not known.

Culmination of signaling at cep-1 is consistent with its essential role in damage-induced apoptosis (Derry et al., 2001). It is at this level that the signaling pathways underlying DNA damage-induced apoptosis interface with those regulating physiological germ cell apoptosis (see above). CEP-1, like other p53 family transcription factors, is phosphorylated in response to DNA damage (Lavin and Gueven, 2006; Quevedo et al., 2007). This activates CEP-1, allowing it to bind to the promoter of the egl-1 gene and activate transcription (Hofmann et al., 2002). The Bcl2 homology domain 3 (BH3)-only protein EGL-1 can bind directly to the Bcl2 homologue CED-9 (Conradt and Horvitz, 1998; del Peso et al., 1998; Fairlie et al., 2006; Parrish et al., 2000), disrupting its ability to inhibit the function of CED-4 in CED-3 activation (del Peso et al., 2000; Yan et al., 2005; Yan et al., 2004). Curiously, egl-1 is required for both damage-induced germ cell as well as developmental apoptosis, but not for physiological germ cell death (Conradt and Horvitz, 1998; Gumienny et al., 1999; Schumacher et al., 2005). This implies that an unidentified pathway regulates CED-9 activity in the setting of physiological germ cell apoptosis (Figure 1.4C). It is also interesting to note that DNA damage-induced germ cell apoptosis does not occur in the male germline (Gartner et al., 2000). Whether male germ cells respond to DNA damage using a similar genetic pathway to direct DNA repair, but not apoptosis, would form an interesting area of study.

1.5 The Phosphatidylinositol 3-kinase Pathway Opposes Apoptosis

Excessive pro-apoptotic signaling can be detrimental to the health of the organism. In neurodegenerative diseases, for example, excessive apoptosis has been implicated in the
functional decline of patients (Bredesen et al., 2006). Pathways have therefore evolved to contest pro-apoptotic signals, a major representative being the phosphatidylinositol 3-kinase (PI3K) pathway. The PI3K pathway is centered on phosphorylated lipid second messengers derived from phosphatidylinositol. Human PI3Ks phosphorylate the 3-position of the inositol ring, using phosphatidylinositol (PtdIns), PtdIns(4)-phosphate, or PtdIns(4,5)-P₂ as a substrate (Figure 1.7A). Which substrate is preferred parses the different PI3Ks into three classes – I, II, and III (Vanhaesebroeck et al., 1997) (Figure 1.7B). It is inferred that *C. elegans* uses the same phosphorylated inositol derivatives as in humans (Morris et al., 1996).

**Figure 1.7 Phosphoinositide chemistry.**

**A)** PI3Ks phosphorylate the 3 position of phosphatidylinositol derivatives. The glycerol backbone and fatty acid tails of the phospholipid moiety are omitted for clarity. Which substrate is preferred depends upon the class of PI3K enzyme (B). Class I enzymes will phosphorylate phosphatidylinositol (PtdIns), PtdIns(4)P, or PtdIns(4,5)P₂ *in vitro*. Class II enzymes prefer PtdIns or PtdIns(4)P *in vitro*, while Class III enzymes restrict themselves to PtdIns as a substrate (Vanhaesebroeck et al., 1997).

Class I PI3Ks have a well-characterized role in anti-apoptotic signaling. These PI3Ks contain a carboxy-terminal lipid kinase domain, preceded by a structurally-important helical domain and
modules that can interact with the small GTPase Ras (rat sarcoma) and an adapter protein known as p85 (Liu et al., 2009). Growth factor binding to membrane-intrinsic receptor tyrosine kinases (RTK) induces their interaction with p85 (Vivanco and Sawyers, 2002) (Figure 1.8A). The resultant RTK-p85 complex then recruits the catalytic subunit of PI3K to the inner side of the plasma membrane, placing the lipid kinase close to its substrate (Didichenko et al., 1996; Klippel et al., 1996). Generation of PtdIns(3,4,5)P₃ in turn concentrates the pleckstrin-homology (PH) domain containing kinases 3-phosphoinositide-dependent protein kinase PDK1 and Akt (non-acronymous) near the site of PI3K action (Alessi et al., 1997a; Anderson et al., 1998; Andjelkovic et al., 1997; Bellacosa et al., 1998; Scheid et al., 2002). This allows PDK1 to directly phosphorylate Akt at Threonine 308 (Alessi et al., 1997a; Alessi et al., 1997b; Scheid et al., 2002; Stephens et al., 1998; Stokoe et al., 1997; Williams et al., 2000).
**Figure 1.8 Phosphatidylinositol 3-kinase signalling in mammals.**

**A** A ligand binds to membrane-intrinsic receptor tyrosine kinases via their ligand-binding domain (LBD). This induces dimerization and autophosphorylation of specific tyrosine residues by the cytoplasmic kinase domains. These phospho-tyrosine (pY) residues recruit the p85 subunit of phosphatidylinositol 3-kinase (PI3K), which binds directly to them. pY-bound p85 can then interact with the catalytic subunit of PI3K (PI3Kcs), p110, inducing the phosphorylation of PtdIns(4,5)P2 [PIP2] to generate PtdIns(3,4,5)P3 [PIP3]. The kinases 3-phosphoinositide-dependent protein kinase 1 (PDK1) and Akt are then recruited to PIP3 via their pleckstrin-homology (PH) domains. This allows PDK1 to phosphorylate Akt at Thr308, facilitating its activation.

**B** Key residues that are phosphorylated during Akt activation. Ser473 is modified by “PDK2”, currently thought to be mTORC2 (Hresko...
Akt is characterized by an amino-terminal PH domain that can directly bind PI3K-generated 3-phosphoinositides (Franke et al., 1997; Frech et al., 1997; Klippel et al., 1997) (Figure 1.8B). Therefore, Akt represents one of the major targets of PI3K signalling (Alessi et al., 1996a; Burgering and Coffer, 1995; Datta et al., 1996; Franke et al., 1995; Kohn et al., 1995). The carboxy-terminal terminus of the protein contains a kinase domain most similar to those present in protein kinases A and C (Bellacosa et al., 1991; Coffer and Woodgett, 1991; Jones et al., 1991a; Jones et al., 1991b). Two key regulatory phosphorylation sites reside within this kinase domain: the aforementioned T308 and another at Ser473 (Alessi et al., 1996a). The phosphorylation of S473 is mediated by a kinase referred to as “PDK2”. Recent searches for this elusive entity have suggested Mammalian Target of Rapamycin in its second complex (mTORC2) as a likely candidate (Hresko and Mueckler, 2005; Jacinto et al., 2006; Sarbassov et al., 2005), but studies in Drosophila have challenged the absolute requirement for TORC2 in Akt function (Hietakangas and Cohen, 2007). In totality, however, phosphorylation of Akt by upstream kinases at these two sites is essential for its complete activity (Alessi et al., 1996a; Andjelkovic et al., 1996; Bellacosa et al., 1998; Kohn et al., 1995; Kohn et al., 1996) since it causes structural re-arrangements in the kinase domain that allow substrate binding and ensure optimal phosphotransfer (Yang et al., 2002a; Yang et al., 2002b).

The convergence upon Akt of many pathways that utilize PI3K is reflected in the number of processes that this kinase affects, including cell growth, proliferation, metabolism and survival (Manning and Cantley, 2007). Akt acts primarily by direct phosphorylation of targets at the consensus site Arg-X-Arg-X-X-S/T-Hyd, where X represents any amino acid and Hyd, bulky, hydrophobic residues (Alessi et al., 1996b; Obata et al., 2000). To oppose pro-apoptotic signals, Akt targets a variety of cell death mediators/effectors including Caspases (Cardone et al., 1998), the BH3-only protein Bad (Blume-Jensen et al., 1998; Datta et al., 1997; del Peso et al., 1997), and the DNA damage transducing kinase Chk1 (homologous to C. elegans CHK-1) (King et al., 2004; Puc et al., 2005; Shtivelman et al., 2002). It (Akt) can also dampen the activity of p53 by
phosphorylating and stabilizing the p53 antagonist Mdm2 (Feng et al., 2004; Gottlieb et al., 2002; Mayo and Donner, 2001; Ogawara et al., 2002; Zhou et al., 2001) and increase the activity of the pro-survival transcription factor nuclear factor-kappa B (NF-κB) by inducing IkappaB kinase (Madrid et al., 2000; Romashkova and Makarov, 1999; Zhou et al., 2000). The swath of cell death modulators that Akt can target reflects its major role in promoting survival (Ahmed et al., 1997; Dudek et al., 1997; Kennedy et al., 1997; Kulik et al., 1997; Philpott et al., 1997; Sabbatini and McCormick, 1999; Songyang et al., 1997) and explains why a number of cancers, including gastric, breast, ovarian, and colorectal contain alterations at the Akt locus that increases its activity (Bellacosa et al., 1995; Brognard et al., 2001; Carpten et al., 2007; Cheng et al., 1996; Kauffmann-Zeh et al., 1997; Nakatani et al., 1999; Ruggeri et al., 1998; Staal, 1987; Sun et al., 2001).

1.6 Phosphatidylinositol 3-kinase Signalling in C. elegans

1.6.1 Developmental Arrest and Lifespan

Owing to its lifestyle as a soil-dweller, C. elegans can be exposed to a variety of adverse environmental stimuli (Brenner, 1974). To endure this adversity, the worm has evolved an alternative larval form - the Dauer - which it can enter under starvation or over-crowded conditions. These larvae can subsequently remain quiescent for extended periods, weathering stress as an alternative form of L3. The return of favourable conditions then stimulates Dauer exit and the resumption of the life cycle at the L4 stage (Hu, 2007). Interestingly, the decision to enter Dauer is controlled by a conserved PI3K signaling pathway (Figure 1.9), composed of the DAF-2 (DAF – abnormal DAuer Formation) RTK (Kimura et al., 1997), the class I PI3K AGE-1 (Morris et al., 1996) (AGE – AGEing alteration), the C. elegans PDK1 homologue PDK-1 (Paradis et al., 1999), and the Akt kinases AKT-1 and AKT-2 (Paradis and Ruvkun, 1998). DAF-2, AGE-1, and PDK-1 activate AKT-1 and AKT-2 (Dorman et al., 1995; Paradis et al., 1999; Paradis and Ruvkun, 1998) and induce them to phosphorylate the forkhead transcription factor DAF-16 (Lin et al., 1997; Lin et al., 2001; Ogg et al., 1997; Paradis and Ruvkun, 1998). This causes phospho-DAF-16 to bind to cytoplasmic 14-3-3 proteins, thereby preventing its entry into the nucleus and preventing the induction of genes required for Dauer entry (Cahill et al., 2001).
Figure 1.9 The *C. elegans* PI3K pathway.

Also referred to as the Insulin-like signalling pathway [owing to homology between DAF-2 and the mammalian Insulin receptor (Kimura et al., 1997)], this pathway controls developmental arrest and lifespan. Mammalian homologues are listed beside *C. elegans* pathway components.

DAF-16 also plays an important role in promoting the lifespan of *C. elegans* and this function is antagonized by components of the worm PI3K pathway, including *daf-2*, *age-1* and *pdk-1* (Larsen et al., 1995; Lithgow et al., 1995; Paradis et al., 1999). Thus, worms with loss-of-function (lf) mutations in *daf-2*, *age-1*, or *pdk-1* live longer than the wild-type - up to twice as long in the case of *daf-2* (Kenyon et al., 1993). The regulation of aging by *akt-1* and *akt-2* is less dramatic, however, suggesting that there are Akt-independent outputs from the *daf-2-age-1-pdk-1* axis in this context (Evans et al., 2008).

1.6.2 Stress Resistance and Innate Immunity

The worm PI3K pathway also plays an important role in regulating the resistance of adult *C. elegans* to stresses such as hypoxia, reactive oxygen species, the accumulation of unfolded
intracellular protein aggregates, and infection by pathogenic bacteria. Generally, loss of daf-2 causes increased DAF-16 activity which facilitates the successful adaption of C. elegans to each of these insults; however, the exact pathway structure leading from daf-2 to daf-16 can vary. In the case of hypoxia and oxidative stress, a pdk-1-akt-daf-16 pathway seems to operate (Scott et al., 2002). Conversely, susceptibility to infection by pathogenic bacteria does not require pdk-1; instead, akt-1 and akt-2 mediate this. Since daf-16 is still required for resistance to pathogens, re-arrangement of the PI3K pathway occurs downstream of pdk-1 in response to infection (Evans et al., 2008). Aggregation of unfolded proteins can have detrimental effects on the viability of individual cells in certain degenerative diseases, such as Alzheimer’s disease in humans (Bredesen et al., 2006). Loss of daf-2 protects C. elegans from death induced by over-expression of the Alzheimer’s disease-linked Aβ peptide (Cohen et al., 2010). This resistance is mediated through increased DAF-16-dependent regulation of chaperone activity, but the signalling intermediates between daf-2 and daf-16 have not been characterized. Thus, a single, conserved PI3K signalling pathway exists in C. elegans to regulate a number of somatic decisions. Whether this pathway has role in antagonizing apoptosis in C. elegans, as PI3K signaling does in mammals, was uncharacterized until recently.

1.6.3 DNA Damage-Induced Germ Cell Apoptosis

Genetic studies by a previous post-doctoral fellow in our group identified a conserved role for akt-1 and akt-2 in promoting the survival of germ cells exposed to DNA damage. Surprisingly, these two kinases were genetically independent from one other and targeted different aspects of the apoptotic signaling cascade: AKT-1 inhibited the phosphorylation and thus, damage-dependent transcriptional function of CEP-1; AKT-2 functioned independently of CEP-1, possibly by regulating the CED-9-CED-4-CED-3 axis (Quevedo et al., 2007) (Figure 1.10).
Figure 1.10 Regulation of damage-induced apoptosis by AKT proteins.

See text for details. Red question mark indicates uncertainty about the exact epistatic position of AKT-2. Mammalian homologues of *C. elegans* pathway components are listed in brackets.

In mammals, one of the key ways that Akt inhibits p53 is through upregulation of Mdm2-dependent p53 degradation (Feng et al., 2004; Gottlieb et al., 2002; Mayo and Donner, 2001; Ogawara et al., 2002; Zhou et al., 2001). AKT-1 regulated CEP-1 independently of E3 ubiquitin ligases, such as an Mdm2 homologue (Quevedo et al., 2007). This suggested that AKT-1 targeted another factor to control CEP-1 phosphorylation. This factor was not *daf-16*, since knockdown of this gene did not affect the response of *akt-1* mutant germ cells to IR (Quevedo et al., 2007). Therefore, it seemed likely that AKT-1 targeted a kinase that controls CEP-1 phosphorylation in response to DNA damage. The PIKKs ATL-1 and ATM-1 were likely targets, but the identity of this intermediary was not established.

At the same time, the independence of *akt-1* from its canonical PI3K pathway target *daf-16* raised the intriguing possibility that there may be other re-arrangements in the worm PI3K pathway in response to DNA damage. Therefore, the DNA damage-specific regulatory mechanisms controlling AKT-1 represented an intriguing area of study. Understanding this process has important implications in cancer since many tumours upregulate Akt signalling to combat chemotherapy (Liu et al., 2009; Vivanco and Sawyers, 2002). Identifying the regulatory pathways that control this phenomenon may allow the design of novel therapeutics that can abrogate oncogenic Akt activity and promote tumour regression. Thus, my aims are to
understand how AKT-1 is regulated in response to DNA damage, and which substrates it targets to inhibit DNA damage-induced *C. elegans* germ cell apoptosis.

In the following, I present data demonstrating a significantly different PI3K pathway structure in the response to DNA damage. The pathway structure involves bifurcations and inversions such as separation of *akt-1* from upstream regulation by *daf-2* and *pdk-1* and inversion of output from *daf-2* and *pdk-1* such that these two regulators promote, rather than inhibit damage-induced cell death. This represents a significant departure from the canonical anti-apoptotic role of mammalian PI3K signalling. In addition, I identify ATL-1 as a potential target for AKT-1, and demonstrate that AKT-1 can inhibit ATL-1 function in multiple ways to dampen pro-death signalling. Together these results define a novel pathway by which *akt-1* regulates damage-induced apoptosis and provide evidence that the structure of the worm PI3K pathway varies significantly between the DNA damage response and the regulation of dauer arrest. Finally, it suggests that novel DNA damage-dependent pathways exist to regulate Akt function *in vivo*. Identifying these pathways will have important implications in cancer therapy.
2 Materials and Methods

2.1 Caenorhabditis elegans Genetics

Worms were maintained on NGM (nematode growth medium) plates seeded with *E. coli* OP50 (Brenner, 1974). Double mutants were constructed according to standard protocols. Alleles used in this study included: Linkage Group I – *atm-1*(gk186) (Stergiou et al., 2007), *cep-1*(gk138) (Derry et al., 2007), *daf-16*(mgDf47) (Ogg et al., 1997), *ppw-1*(pk1425) (Tijsterman et al., 2002), *rrf-1*(pk1417) (Sijen et al., 2001); Linkage Group II – *age-1*(ag12) (Miyata et al., 2008), *age-1*(hx546) (Tissenbaum and Ruvkun, 1998), *age-1*(mg44) (Morris et al., 1996), *let-23*(n1045) (Aroian et al., 1993), *let-23*(sy1) (Aroian et al., 1994), *let-23*(sy12) (Sakai et al., 1996), *let-23*(sy97) (Aroian et al., 1994), *lin-7*(e1449) (Simske et al., 1996), *rrf-3*(pk1426) (Simmer et al., 2002); Linkage Group III – *ced-4*(n1162) (Yuan and Horvitz, 1992), *ced-4*(n2273) (Yuan and Horvitz, 1992), *ced-9*(n2812) (Hengartner and Horvitz, 1994), *ckk-1*(ok1033) (Wormbase), *ckk-1*(tm421) (Kimura et al., 2002), *daf-2*(e1370) (Kimura et al., 1997), *daf-2*(e1391) (Kimura et al., 1997), *daf-2*(m596) (Patel et al., 2008), *RICT-1*(fit7) (Jones et al., 2009), *RICT-1*(mg360) (Jones et al., 2009), *W04B5.5*(ok1309) (Wormbase), *W04B5.5*(tm2668) (Wormbase); Linkage Group V – *akt-1*(ok525) (Hertweck et al., 2004), *akt-1*(ok525); byEx[akt-1::GFP] (Hertweck et al., 2004), *atl-1*(tm853) (Garcia-Muse and Boulton, 2005), *egl-1*(n1084n3082) (Conradt and Horvitz, 1998); Linkage Group X – *akt-2*(ok393) (Hertweck et al., 2004), *pdk-1*(mg142) (Paradis et al., 1999), *pdk-1*(sa680) (Paradis et al., 1999), *pdk-1*(sa709) (Paradis et al., 1999), *sli-1*(sy143) (Jongeward et al., 1995).

2.2 Quantification of Germ Cell Apoptosis

Worms were picked at the L4 stage - as judged by nascent formation of the hermaphrodite vulva (Lewis and Fleming, 1995) - aged the desired amount and then subjected to ionizing radiation from a $^{137}$Cs source, or ultraviolet (UV) radiation from a Stratagene 1800 UV Crosslinker. For UV irradiation, worms were first transferred to an unseeded NGM plate, then irradiated with the plate top removed, and finally returned to seeded plates for recovery. After irradiation, all worms were incubated for fixed time/temperature intervals until analysis. To observe apoptotic cells, worms were mounted on 3-4% agarose pads on glass slides overlaid with a cover slip.
mM final concentration of L-tetramisole in M9 buffer (Brenner, 1974) was included to paralyze body wall muscles and one germline arm in each animal was observed on a Leica DMRA2 system using standard Differential Interference Contrast (DIC) optics. Apoptotic cells were distinguished by their characteristic raised and highly refractile morphology (Gumienny et al., 1999; Kerr et al., 1972). To confirm the apoptotic nature of cells, they were stained with acridine orange (AO), a DNA binding dye that shows preference for dying cells (Abrams et al., 1993) as follows: plates seeded with OP50 were flooded with 75 μg/mL of AO in M9 buffer and allowed to dry for 2 hours at room temperature in the dark; worms were placed on the dried plates for 2 hours and allowed to feed on stained bacteria in the dark; to purge excess AO, worms were picked to fresh OP50-seeded plates lacking AO and allowed to feed for 2 hours in the dark. Apoptotic cells staining with AO were visualized using standard fluorescence optics and confirmed by sequential examination in the DIC channel.

2.3 Statistical Analyses

Statistical significance was determined in Microsoft Excel using a one- or two-sided Student’s t-test, assuming equal variance.

2.4 Quantification of Germ Cell Number

L4 stage worms were incubated at 25°C for 24 hours and then washed from plates in M9 buffer. Pelleted worms were subsequently fixed in methanol for at least 5 minutes at -20°C. Fixed worms were washed once with phosphate-buffered saline containing 0.1% v/v Tween-20 (PBST), stained with 1 μg/mL DAPI (4′,6-diamidino-2-phenylindole) in PBST for 30 minutes in the dark, cleared of excess DAPI by one wash in PBST, and then mounted directly onto glass slides. Pachytene nuclei were identified in whole worms by their distinctive chromosomal morphology using standard epifluorescence filters. Scanning images throughout the entire thickness of the germline were recorded with a Hamamatsu CCD camera and total pachytene nuclei in one gonad arm of each worm were quantified using Openlab software (PerkinElmer).
2.5 Quantification of Apoptotic Cell Engulfment in \textit{pdk-1(mg142)}

L4 stage worms were irradiated with 60 Gy and then incubated for 24 hours at 25°C. At this point, wild-type and \textit{pdk-1(mg142)} worms were mounted abreast on a single slide and serial images were taken through the entire thickness of the gonad using recorded set points in the Openlab software. Images were acquired every 15 minutes for 2.5 hours. The latency of an apoptotic cell began the moment that changes in refractility made it distinguishable from neighbours and ended upon disappearance of the nucleus. Ten apoptotic cells of each genotype were examined to obtain the average latency in a given strain.

2.6 RNA Interference

All RNA interference (RNAi) was performed by feeding (Timmons et al., 2001; Timmons and Fire, 1998) according to the method of Kamath \textit{et al.} (2001). Briefly, bacterial cultures expressing double-stranded RNA targeting the gene of interest were drawn from the Ahringer library (Kamath \textit{et al.}, 2003), unless otherwise noted. They were grown in liquid culture for 8 hours at 37°C and seeded onto NGM plates containing 25 μg/mL carbenicillin and 2.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) to allow induction overnight at room temperature. Worms were fed RNAi from the L1 stage. Control RNAi was drawn from clone \textit{Y95B8A_84.g}, which targets a non-expressed gene (Lehner \textit{et al.}, 2006). The \textit{daf-2} RNAi clone targeting exon 14, generated as described (Dixon \textit{et al.}, 2008), was a kind gift of Peter Roy (University of Toronto).

2.7 Quantification of CEP-1/p53 Activity

Induction of \textit{egl-1} was quantified following irradiation (see above) by lysing treated worms in Trizol (Invitrogen). RNA was purified according to the manufacturer’s protocol. 1-4 μg of RNA (as judged spectrophotometrically) was subjected to reverse transcription with SuperScript III reverse transcriptase using an Oligo(dT) primer, according the manufacturer’s protocol (Invitrogen). cDNA was diluted 1:40 in diethylpyrocarbonate-treated ddH2O and quantified as described (Quevedo \textit{et al.}, 2007) using a Roche LightCycler 480 Real Time PCR system with
Roche SYBR Green I Master mix. The amount of egl-1 mRNA present in each sample was determined by relative quantification using a standard curve assembled from internal control data. tbg-1 mRNA was used to normalize egl-1 levels and data were plotted relative to the wild-type unirradiated control.

2.8 Generation of AKT-1 Antibodies

Two goat mono-specific polyclonal antisera against *C. elegans* AKT-1 (128 and 527) were generated by Bethyl Laboratories (Montgomery, Texas). Peptide antigens corresponded to residues 128-145 (QEELMETNQQPKIDEDSE), which is part of the linker region between the PH and kinase domains of AKT-1, and residues 527-541 (RIHEASEDNEDYDMG), which represents the C-terminus of the protein. Both antisera independently recognized a doublet at 70 kDa on Western blots of whole worm lysates, which was absent from the genetic null *ok525* (Hertweck et al., 2004). This is slightly larger than the predicted mass of 62 kDa; the reason for this discrepancy is not known.

2.9 Immunoprecipitation and Western Blotting

Worms of the indicated genotype were synchronized by hypochlorite treatment (Lewis and Fleming, 1995) and grown at 15°C to the L4 stage as described for apoptosis assays. Worms were then irradiated with the indicated dose of IR as described for apoptosis assays and then incubated for 24 hours at 20°C. After washing worms from plates with PBS, the pellets were washed once more with PBS and then quickly snap frozen in liquid nitrogen. Approximately 500 µL of Lysis Buffer [25 mM Tris (pH 7.4), 1% v/v Triton X-100, 10% v/v glycerol, 150 mM NaCl, 25 mM β-glycerophosphate, 2 mM phenylmethanesulfonylfluoride (PMSF)] supplemented with Phosphatase Inhibitor Cocktails I and II (Sigma) and Protease Inhibitor Cocktail (Roche) was added to the worm pellet and sonicated with a Misonix (3000) sonicator (power output of 4 for 10 seconds each with ~1 minute rest between pulses until the worms were completely lysed). The lysates were then clarified by centrifugation at 13,000 rpm for 10 minutes at 4°C and the protein content was estimated by Quick Bradford (Pierce). A mixture of approximately 2.5 µg each of the two AKT-1 128 and 527 antibodies was used to
immunoprecipitate AKT-1 from 0.6–1mg of total protein (equal amounts of total protein were used in a given experiment) and antigen-antibody complexes were allowed to form overnight at 4°C with rotation. The antigen-antibody complex was subsequently captured with 50 µL of protein-G agarose beads (Upstate) for 2 hours at 4°C with rotation. Immune complexes bound to beads were washed 3 times with Lysis Buffer supplemented with phosphatase and protease inhibitors and finally boiled in Laemmli sample buffer [0.0625 M Tris (pH 6.8), 2% w/v SDS, 10% v/v glycerol, 5% v/v 2-mercaptoethanol, 0.002% w/v bromophenol blue].

Immunoprecipitated proteins were separated by SDS-PAGE on a 10% resolving gel, transferred to polyvinylidene fluoride membranes, and blocked in Tris-buffered saline-0.1% v/v Tween-20 (TBST) containing 5% skim milk (T350 and total AKT-1 antibodies) or 5% BSA (S517 antibody). Blots were then probed with rabbit phospho-specific antibodies directed against AKT-1 T350 (Padmanabhan et al., 2009) diluted 1:1000 in TBST containing 5% BSA, followed by anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000 in TBST containing 5% skim milk). After exposure, secondary antibodies were inactivated with 0.1 N HCl and the membrane was reprobed for total AKT-1 using a 1:1 mixture of goat AKT-1 antibodies 128 and 527 diluted 1:16,000 in TBST containing 5% skim milk, followed by anti-goat HRP-conjugated secondary antibodies (1:5000 in TBST containing 5% skim milk). The antibodies were then stripped from the membrane using a 6M guanidine hydrochloride solution as described (Yeung and Stanley, 2009). After blocking again in TBST containing 5% BSA, the membrane was probed for S517 phosphorylation using rabbit phospho-specific antibodies targeting AKT-1 S517 (Padmanabhan et al., 2009) diluted 1:750 in TBST containing 5% BSA. Secondary antibodies were as described for the T350 site. Sometimes, when sufficient protein was isolated, T350 and S517 phosphorylation were analyzed on different membranes by probing each with phospho-specific antibodies first, and then stripping the membrane as described above, and finally probing for total AKT-1.

2.10 Immunostaining

2.10.1 Dissected Germlines

Young adult worms (24 hours post-L4 stage) were treated with IR as described above and incubated for 24 hours at 20°C (for AKT-1 immunostaining) or 25°C (for CED-4
immunostaining). Worms were washed from plates with three washes of M9 buffer and the pellet was washed once with 0.01% v/v Tween 20 in M9 and once with ddH2O. The supernatant was aspirated save ~ 50 μL and, using a pasteur pipette, worms were dropped onto slides pre-coated with polylysine. After brief settling, excess liquid was drawn off. Germlines were then dissected from ~50 worms using a 25 gauge needle in ~30 μL phosphate-buffered saline (PBS). PBS was then replaced with ~50 μL of fresh 2% w/v paraformaldehyde (PFA) and germlines were fixed for 5 minutes. After fixing, all but ~8 μL of PFA was removed and slides were overlaid with a glass coverslip and placed on dry ice (on top of a flat metal plate that had been precooled) for 2-3 minutes. Coverslips were then “flicked” off and freeze-cracked germlines were post-fixed for 2 minutes at -20°C in 50:50 mixture of methanol:acetone.

To stain, germlines were permeabilized twice for 10 minutes with 1% v/v Triton X-100 in PBS. Background staining was reduced by incubating in Image-iT FX Signal Enhancer (Invitrogen) for 20 minutes at room temperature and then slides were blocked in 1% w/v BSA or 10% normal Donkey serum (NDS) in PBST for 30 minutes. To detect CED-4 and AKT-1, germlines were stained with 1:300 α-CED-4 (9104) (Greiss et al., 2008) and 1:200 Nuclear Pore Complex Proteins mAb414 (Abcam) antibody (as a protein loading control), or 1:100 α-AKT-1 (generated in this study), respectively, overnight in a humid chamber with parafilm covering the germlines. Slides were washed three times for 15 minutes each with PBST, stained with a 1:500 dilution of Alexa-conjugated goat α-rabbit and 1:400 Alexa-conjugated goat α-mouse (CED-4) or 1:500 Alexa-conjugated donkey α-goat (AKT-1) in PBST plus BSA or NDS for 1 hour, and then washed a further three times, for 15 minutes each, with PBST. DNA was stained with 1 mg/mL DAPI in PBST for 15 minutes and after, slides were washed again with PBST. Slides were then mounted in ProLong Gold (Invitrogen) and visualized using standard epifluorescence filters on a Leica DMRA2 system. Pictures were acquired with a Hamamatsu CCD camera and Openlab software (PerkinElmer) before processing in Adobe Photoshop and Illustrator. All staining steps were performed at room temperature, unless otherwise noted.
2.10.2 Whole Worms

Worms were grown, irradiated, washed from plates as described above. Instead of immediate fixation after harvest, worms were freeze-cracked on polylysine-coated slides in ddH2O and then were fixed in 4% w/v formaldehyde in PBS for 1 hour. Slides were washed once with PBST, taking care to collect detached worms in a 1.5 mL microfuge tube. Worms were then post-fixed in 50:50 methanol:acetone for 5 minutes at -20ºC and washed three times with PBST for 10 minutes each. Non-specific signal was blocked in Buffer A [1% w/v BSA, 0.5 % v/v Triton X-100, 5 mM NaN3, 1 mM disodium ethylenediaminetetraacetate (EDTA) in PBS] and then worms were stained with a 1:100 dilution of either 128 or 527 AKT-1 antiserum, or a mixture of both in Buffer A in a humid chamber as described for dissected germlines. Unbound primary antibodies were removed by three washes with Buffer B (same as Buffer A, except 0.1% w/v BSA) for 10 minutes each and then stained with 1:500 Alexa-conjugated donkey α-goat for 3 hours. A final set of three washes with Buffer B and staining with 1 mg/mL DAPI was performed before mounting worms for visualization, as described above. Worms that detached from the slide after the formaldehyde fixation step were stained in microfuge tubes using the above protocol and subsequently mounted on slides for visualization. Staining patterns generated in tubes were identical to those on slides. All staining steps were performed at room temperature, unless otherwise noted.
3 Results 1: DNA Damage-Dependent Regulation of AKT-1/Akt

3.1 Data Attribution

I would like to acknowledge Ashley Ross for helping to generate the ced-4(n1162); pdk-1(mg142) double mutant used in Figure 3.4F. Bin Yu performed the CED-4 immunostaining presented in Figure 3.5 and assisted immensely in the initial optimization of AKT-1 immunostaining, immunoprecipitation and *in vitro* kinase assays. I also express my gratitude to Kelvin Yen for performing the phospho-AKT-1 immunoblotting presented in Figures 3.8 and 3.9A. All other data were acquired by the author.

3.2 PI3K Signalling Elements Promote DNA Damage-induced Germ Cell Apoptosis

Based on published literature regarding the role of *akt-1* in dauer arrest, I hypothesized that the DNA damage-dependent function of AKT-1 would be controlled by the components of the *C. elegans* PI3K pathway – DAF-2, AGE-1 and PDK-1 (Figure 3.2A). If this were true, loss-of-function (lf) mutations in *daf-2*, *age-1*, and *pdk-1* should generate a similar apoptotic phenotype to that of *akt-1* null mutants (*i.e.* a strong sensitivity to DNA damage-induced germ cell apoptosis). To test this, I exposed L4-stage *daf-2(e1370)* worms to ionizing radiation (IR), shifted the worms to 25°C, and then counted the number of apoptotic germ cells that formed 24 hours later. Unexpectedly, DNA damage-induced apoptosis was strongly suppressed in the germline of *daf-2(e1370)* mutants (Figure 3.2B). This directly contradicted previous studies, which reported a weak anti-apoptotic role for *daf-2* in damage-induced apoptosis (Pinkston-Gosse and Kenyon, 2007; Pinkston et al., 2006). To determine if my observations with the *e1370* allele were the result of background mutations in the strain used, I confirmed that a similar phenotype resulted using two other lf alleles of *daf-2* - *e1391* and *m596*. These two mutations affect widely separated domains of the DAF-2 protein: the intracellular kinase domain in *e1391* and the extracellular ligand-binding domain in *m596* (Patel et al., 2008) (Figure 3.1). My ability to replicate resistance to damage-induced apoptosis in the two other missense alleles of *daf-2* strongly suggested that *daf-2* is required for damage-induced apoptosis (Figure 3.2B).
Figure 3.1 Structure of DAF-2/InsR and location of daf-2 mutations used in this study.

Left, daf-2 encodes a large transmembrane receptor tyrosine kinase with homology to the vertebrate Insulin receptor (InsR) (Kimura et al., 1997). It is a homo-dimeric receptor composed of two monomers that are in turn composed of an α and a β chain. Disulphide bonds link both the α and β chains within each monomer and the two monomers in the holo-receptor (red lines). The extracellular domain of DAF-2 consists of, from amino to carboxyl termini, Leucine- and Cysteine-rich domains as well as multiple Fibronectin type III (FnIII) repeats. Ligand binding is conferred by the amino-terminal leucine-rich repeat and the cysteine-rich domain, with minor contributions from the carboxyl-terminal FnIII repeat of the α-chain (Ward et al., 2007). The cytoplasmic tyrosine kinase domain is followed by an Src homology domain 2 (SH2)-binding domain that contains multiple tyrosine residues that can be
autophosphorylated and subsequently bind to the p85 subunit of PI3K or other adaptor proteins, such as Insulin receptor substrate protein 1 (IRS1). Right, the locations of the daf-2 mutations used in this study. Adapted from (Patel et al., 2008).

To test whether the resistance to apoptosis observed in daf-2 mutants also occurred when other PI3K pathway components were mutated, I examined IR-induced apoptosis in strains homozygous for if alleles in age-1, and three additional alleles of pdk-1. The counts were confirmed with the DNA binding dye acridine orange, which stains the condensed chromatin present in apoptotic cells (Abrams et al., 1993). In accord with daf-2, null and If mutations in either age-1 or pdk-1 also generated strong resistance to IR-induced germ cell apoptosis (Figure 3.2C-E). The gain-of-function (gf) mg142 allele of pdk-1 had the opposite effect (i.e. hypersensitivity to DNA damage), and because this was not caused by the delayed or defective clearance of apoptotic cells in pdk-1(mg142) (Table 3.1), I confirmed that age-1 and pdk-1 are both required to promote, rather than inhibit, damage-induced germ cell apoptosis.
Figure 3.2 PI3K signalling components promote DNA damage-induced germ cell apoptosis.

A) The PI3K pathway in *C. elegans*. B-E) *C. elegans* PI3K signalling components are required for damage-induced apoptosis. B-C, E) L4-stage worms were treated with IR and shifted to 25°C. Germ cell apoptosis was quantified 24 hours later. *pdk-1(0) = pdk-1(sa680), pdk-1(lf) = pdk-1(sa709), pdk-1(gf) = pdk-1(mg142).* D) Same as above, except that *age-1(mg44) [age-1(0)]* worms were incubated at 15°C after IR. Because the kinetics underlying apoptosis are increased at higher temperatures, the number of apoptotic cells observed in assays conducted at 25°C is
higher than that seen at 15°C. This reflects temperature-dependent effects on the apoptotic process itself; the relative difference between genotypes is usually independent of this. Error bars represent the standard error of the mean (SEM) from at least three independent experiments. At least ten animals were examined in each experiment.

### Table 3.1 pdk-1 does not regulate the clearance of apoptotic cells

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Average Corpse Latency ± SD (min)</th>
<th>N</th>
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</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>48.0 ± 24.3</td>
<td>10</td>
</tr>
<tr>
<td>pdk-1(mg142)</td>
<td>49.1 ± 31.5</td>
<td>11</td>
</tr>
</tbody>
</table>

L4-stage worms were treated with 60 Gy of ionizing radiation and then incubated at 25°C for 24 hours. Persistence of apoptotic cells (corpses) was monitored by time-lapse microscopy, using the onset of refractility as the initiating event and the disappearance of the cell as the closing. SD = standard deviation.

Because genetic data in *Drosophila* have implicated Insulin signalling in the control of germ cell proliferation (LaFever and Drummond-Barbosa, 2005), I wanted to ensure that reductions in germ cell proliferation in *daf-2* and *pdk-1* did not lead to reduced capacity to execute apoptosis. Therefore, I counted DAPI-stained pachytene nuclei in *daf-2(e1370)* and *pdk-1(sa680)* [a predicted null mutation (Paradis et al., 1999)] germlines 24 hours after shifting L4-stage worms to 25°C. Under my assay conditions, both mutants contained approximately 80% of the wild-type number of pachytene germ cells (Table 3.2). While this represents an absolute decrease in germ cell number of 20%, it is insufficient to explain the ~80% decrease in the levels of damage–induced apoptosis in *daf-2(e1370)* and *pdk-1(sa680)* mutants when compared to the wild-type (Figures 3.2B,E).
## Table 3.2 daf-2 and pdk-1 have a minor role in germline proliferation

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of Pachytene Nuclei ± SD</th>
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<tbody>
<tr>
<td>wild-type</td>
<td>334 ± 49</td>
<td>10</td>
</tr>
<tr>
<td>daf-2(e1370)</td>
<td>268 ± 37</td>
<td>10</td>
</tr>
<tr>
<td>pdk-1(sa680)</td>
<td>260 ± 67</td>
<td>10</td>
</tr>
</tbody>
</table>

Young adult worms (24 hours post L4 stage at 25°C) were methanol-fixed and stained with DAPI to identify pachytene nuclei. For each animal observed, only one gonad arm was scored.

Since all of my apoptosis assays were performed at similar stages of development and using identical temperature shift protocols, it is unlikely that the apoptosis defects that I observe in daf-2 and pdk-1 mutants results from defective germline proliferation. However, I did observe a strong germline proliferation defect in the age-1 null mutant mg44 (unpublished observations); for fear of confounding effects, I did not characterize age-1 further. Since daf-2 and pdk-1 have opposing effects on apoptosis to those of akt-1, I was interested in determining whether the linear structure of the worm PI3K pathway (Figure 3.2A) was preserved in the context of this signalling inversion.

### 3.3 daf-2/InsR and pdk-1/PDK1 Do Not Function Upstream of akt-1/Akt

If akt-1 is antagonized by daf-2 in a linear pathway, null mutations in akt-1 should be able to revert the resistance to apoptosis of daf-2 mutants. To test this directly, I constructed a daf-2(e1370); akt-1(ok525) double mutant (ok525 is a protein null allele of akt-1; Figure 3.8). Unfortunately, I could not quantify germ cell apoptosis in this strain since these worms were extremely long and thin at all temperatures, which impeded my ability to visualize the gonad. To circumvent the apparently additive somatic effects of deleting daf-2 and akt-1 (Padmanabhan et al., 2009), I knocked down daf-2 by RNA interference (RNAi) instead. Fortunately, this faithfully recapitulated the resistance to apoptosis seen in daf-2 mutants and did not generate adverse somatic phenotypes in akt-1(0) mutants. This allowed me to study the epistasis between daf-2 and akt-1. Unexpectedly, loss of akt-1 did not revert the resistance of daf-2(RNAi) germ
cells to DNA damage (Figure 3.3A). This was not consistent with a linear model of PI3K signalling and instead suggested that *daf-2* functions either downstream, or independently of, *akt-1*.

This directly contradicted contemporary models of PI3K signalling and thus, I felt it prudent to examine the structure of the worm PI3K pathway further in *akt-1(0); pdk-1(0)* double mutants. Initially, I found it exceedingly difficult to generate *akt-1(0); pdk-1(0)* double mutants since I was unable to obtain cross-progeny from matings using *pdk-1(sa680)* worms. I was able to generate *pdk-1(sa680)* males by heat-shock, but these would not produce cross-progeny with isogenic *sa680* hermaphrodites, nor allogenic *akt-1(0)* or wild-type hermaphrodites. Similarly, *sa680* hermaphrodites did not generate cross-progeny when mated with wild-type, or either zygosity of *akt-1(0)* males (unpublished observations). My ability to simultaneously generate adult *pdk-1(sa680)* males and to not mate *pdk-1(sa680)* hermaphrodites is at odds with data reported by Paradis and colleagues (1999). They instead found that *pdk-1(sa680)* males arrested as Dauer or earlier stage larvae at all temperatures and that *pdk-1(sa680)* hermaphrodites were able to mate efficiently enough with wild-type males to facilitate outcrossing. Since I verified the presence of the *sa680* mutation in my *pdk-1(sa680)* strain phenotypically before using it in heat-shocks (unpublished data), it is strange that I could not mate *pdk-1(sa680)* worms [*sa680 causes constitutive dauer arrest at 25°C (Paradis et al., 1999)*]. Perhaps different mating methodologies underlie these inconsistencies.

To circumvent these difficulties I turned to the *sa709* hypomorphic allele (Paradis et al., 1999) and tried to construct an *akt-1(0); pdk-1(sa709)* double mutant; I also attempted to knockdown *pdk-1* by RNAi, but this was not penetrant [data not shown and (Paradis et al., 1999)]. Contrary to the case with *sa680*, I was able to generate an *akt-1(0); pdk-1(sa709)* strain. When I treated these worms with IR, I found that, as in the case of *daf-2*, loss of *akt-1* was unable to revert the *pdk-1* dependent resistance to apoptosis (Figure 3.3B). The fact that a hypomorphic mutation in *pdk-1* can fully suppress *akt-1(0)* levels of cell death strongly suggests that *pdk-1* does not function upstream of *akt-1*. Combined, my data represent the first demonstration of a significant re-arrangement in the worm PI3K pathway, with *akt-1* functioning upstream or independently of *daf-2* and *pdk-1*, instead of downstream.
3.3 daf-2 and pdk-1 do not function upstream of akt-1.

A) Young adults were irradiated, incubated at 20°C for 24 hours, and apoptosis was quantified as in Figure 1. RNAi was performed in the rrf-3(pk1426) background. akt-1(0) = akt-1(ok525). B) L4 stage worms were treated as in A. pdk-1(lf) = pdk-1(sa709), akt-1(0) = akt-1(ok525). Error bars as in Figure 3.2.

3.4 daf-2/InsR and pdk-1/PDK1 Do Not Function as Sensors of DNA Damage

To understand this re-organization of the worm PI3K pathway, I wanted to determine how daf-2 and pdk-1 regulate apoptosis. In the C. elegans germline, two parallel pathways function to sense DNA damage and transmit it to cep-1/p53: the first is encoded by the C. elegans homologues of the mammalian 9-1-1 complex, hpr-9, mrt-2 and hus-1 (Ahmed and Hodgkin, 2000; Hofmann et al., 2002); the second is mediated by the clk-2 gene product (Ahmed et al., 2001). By activating the DNA damage-dependent kinases ATL-1 and ATM-1 (homologous to mammalian ATR and ATM proteins, respectively), hpr-9-mrt-2-hus-1 and clk-2 transmit the presence of damage to CEP-1/p53 (Hofmann et al., 2002). This activates CEP-1 and allows it to transactivate the egl-1 promotor. Expressed EGL-1 then binds to the Bcl2 homologue CED-9 (Conradt and Horvitz, 1998), freeing CED-4/Apaf1 to activate the Caspase CED-3 (Shaham and Horvitz, 1996). Activation of CED-3 is one of the major initiating events of germ cell apoptosis (Gumienny et al., 1999) (Figure 3.4A).
Although in response to DNA damage germ cells die in the pachytene region of the germline, mitotic germ cells in the distal tip can also transiently arrest (Gartner et al., 2000). Mutations in the DNA damage sensor genes \textit{hpr-9}, \textit{mrt-2}, \textit{hus-1}, and \textit{clk-2} cause defects in this mitotic cell cycle arrest and also prevent apoptosis in the pachytene region (Ahmed et al., 2001; Gartner et al., 2000). Because loss of \textit{daf-2} and \textit{pdk-1} significantly reduced damage-dependent apoptosis of pachytene germ cells (Figures 3.2B,E), I wanted to know whether these defects were also accompanied by a failure to arrest the germ cell cycle in the distal tip. Therefore, I treated \textit{daf-2} and \textit{pdk-1} mutants with IR and examined the size of nuclei in the mitotic zone of the germline. Wild-type nuclei increase in size as they arrest and I saw no difference between wild-type and \textit{daf-2} and \textit{pdk-1} mutant nuclei in this context (unpublished observations). This damage-induced cell cycle arrest in the distal germline is inferred to allow repair of damaged DNA (Ahmed et al., 2001; Gartner et al., 2000). Mutations that compromise this arrest are accompanied by defects in apoptosis and decreased survival of progeny produced from irradiated germ cells (Gartner et al., 2000). While \textit{lf} mutations in \textit{hpr-9}, \textit{mrt-2}, \textit{hus-1}, and \textit{clk-2} lead to increased levels of embryonic lethality after irradiation when compared to wild-type, the progeny of \textit{daf-2(e1370)} worms display less than wild-type levels of embryonic lethality after irradiation (Luo et al., 2010). This further supports my contention that \textit{daf-2} and \textit{pdk-1} do not function as sensors of DNA damage and that they are instead required to promote apoptosis downstream of, or in parallel to, \textit{hpr-9-mrt-2-hus-1} and \textit{clk-2}.

### 3.5 \textit{daf-2}/InsR and \textit{pdk-1}/PDK1 Function Independently of \textit{cep-1}/p53, Downstream of \textit{akt-1}/Akt

\textit{akt-1} controls DNA damage-induced cell death through \textit{cep-1}, such that null mutations in \textit{akt-1} cause excessive CEP-1-dependent \textit{egl-1} induction and apoptosis, while \textit{gf} mutations in \textit{akt-1} generate the converse (Quevedo et al., 2007). I reasoned that if \textit{daf-2} and \textit{pdk-1} functioned downstream of \textit{akt-1}, but upstream of \textit{cep-1}, their loss would impede the activation and subsequent transcriptional function of CEP-1. On the contrary, if they functioned independently of \textit{akt-1}, then CEP-1–dependent \textit{egl-1} induction would be unaffected. To assess this, I treated worms homozygous for multiple alleles of \textit{daf-2} and \textit{pdk-1} with IR, shifted the worms to the restrictive temperature for 24 hours and then assessed CEP-1 activity by Real Time PCR. In
response to DNA damage, wild-type worms induced egl-1 approximately 5-fold while cep-1 mutants did not induce egl-1 at all (Figure 3.4B). Surprisingly, egl-1 was induced to nearly twice the wild-type level in daf-2(e1370) and daf-2(m596) mutants. This suggested that daf-2 was not required for CEP-1-dependent egl-1 induction, but might actually inhibit CEP-1 activity in response to IR. Further characterization demonstrated that loss of pdk-1, in the sa680 null or in the hypomorph sa709 did not affect egl-1 induction, and neither did the mg142gf mutation (Figure 3.4B). Because the effect of IR on egl-1 in daf-2 and pdk-1 mutants is entirely dependent on cep-1 (Figure 3.4B), daf-2 and pdk-1 are not required for CEP-1 transcriptional function. The fact that CEP-1 still receives activating signals in daf-2 and pdk-1 mutants and yet germ cells still cannot die, demonstrates that daf-2 and pdk-1 promote apoptosis downstream, or in parallel to, cep-1.
Figure 3.4 *daf-2* and *pdk-1* regulate the core apoptosis pathway independently of *cep-1/p53* and *akt-1*.
A) Genetic pathway controlling DNA damage-induced germ cell apoptosis. See text for details. B) L4 stage worms were treated with IR, incubated at 25°C for 24 hours, and total RNA was isolated. *egl-1* transcript was quantified by Real Time PCR using *tbg-1* (*tbg – TuBulin, Gamma*) as an internal standard. *cep-1(lf) = cep-1(gk138), pdk-1(0) = pdk-1(sa680), pdk-1(lf) = pdk-1(sa709), pdk-1(gf) = pdk-1(mg142)*. Error bars represent the SEM from at least three independent experiments. C-F) *daf-2* and *pdk-1* regulate the core apoptosis pathway through *ced-9* and *ced-4*, respectively. L4 stage (C,E,F) or young adult (D) worms were irradiated and germ cell apoptosis was quantified after 24 hours at 20°C (D,E), or 25°C (C,F). *daf-2(lf) = daf-2(e1370), ced-9(0) = ced-9(n2812), ced-4(0) = ced-4(n1162).* Error bars as in Figure 3.2.

### 3.6 *daf-2*/InsR and *pdk-1*/PDK1 Function in Mutually Exclusive Pathways to Drive Cell Death Independently of *cep-1*/p53

The ability of *daf-2* and *pdk-1* to function in a similar way to regulate damage-induced apoptosis raised the possibility that a linear *daf-2-pdk-1* pathway functioned in parallel to *cep-1*. To test this directly, I examined whether an increase PDK-1 activity was able to revert the resistance to damage-induced germ cell apoptosis in *daf-2* mutants (Figure 3.4C). Because *pdk-1(mg142)* was unable to revert the apoptosis defect in *daf-2(e1370)* mutants, it seems that *pdk-1* is not a major downstream target of *daf-2*. The fact that the *mg142* allele was originally isolated as a dominant suppressor of *age-1* and not *daf-2*, and the fact that *mg142* possesses only a limited ability to suppress the constitutive dauer arrest of *daf-2(e1370)* (Paradis et al., 1999) suggests that this analysis could be confounded. Furthermore, it is possible that although the *mg142* mutation does not require AGE-1 activity in the context of dauer arrest (Paradis et al., 1999), PDK-1(mg142) could require AGE-1 in response to DNA damage. To verify the independence of *daf-2* and *pdk-1*, I further characterized at which level they both act in the pathway controlling damage-induced germ cell apoptosis.

Downstream of CEP-1-dependent *egl-1* induction, CED-9/Bcl2 controls the activity of CED-4/Apaf1 in CED-3/Caspase activation (Chinnaiyan et al., 1997b; James et al., 1997; Otitilie et al., 1997; Spector et al., 1997; Wu et al., 1997; Yan et al., 2005) (Figure 3.4A). To determine if *daf-2* and *pdk-1* functioned at the level of *ced-9*, I assessed whether loss of either could suppress the excessive germ cell apoptosis in *ced-9* null mutants. Informatively, loss of *daf-2* did not alter germ cell apoptosis in *ced-9(n2812)* null mutants, while loss of *pdk-1* reduced apoptosis partially, although this was not statistically significant either in the presence or absence of IR
Because of the somatic effects of *daf-2* and *pdk-1* loss on the visibility of the germline in *daf-2ced-9* and *ced-9; pdk-1* double mutants, I had to perform these two experiments at different developmental times (48 hours post-L4 in the case of *daf-2ced-9* and 24 hours post-L4 in the case of *ced-9; pdk-1*). As worms age from L4 to young adult, the germline continues to expand in size and this is reflected by a slight increase in basal levels of apoptosis (Quevedo et al., 2007). Therefore, the levels of physiological apoptosis seen in *ced-9(0)* mutants in the *daf-2ced-9* experiment is greater than that seen in the *ced-9; pdk-1* experiment. This should not alter my interpretation of epistasis, however, since loss of *pdk-1* causes an ~1.3-fold greater reduction in *ced-9(0)*-dependent apoptosis than does loss of *daf-2*. Since loss of *daf-2* and *pdk-1* have identical effects on germline proliferation (Table 3.2), my data suggest that the partial reduction of apoptosis in *ced-9(0)* by caused by *pdk-1(sa709)* reflects a true regulatory relationship. Therefore, *daf-2* may function upstream, or independently of *ced-9*, while the additive effects on apoptosis of *pdk-1(sa709)* and *ced-9(n2812)* could reflect the functioning of *pdk-1* downstream or independently of *ced-9*.

*pdk-1* does not appear to function directly downstream of *ced-9*, however, since *pdk-1(sa709)* was unable to rescue the embryonic lethality and sterility of maternally-rescued *ced-9(0)* homozygotes (Table 3.3) - mutations in *ced-3* and *ced-4*, which function strictly downstream of *ced-9*, are able to accomplish this (Hengartner et al., 1992).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Brood Size (N=3)</th>
<th>% Eggs Hatching</th>
<th>% Reaching L4/Adult</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>272.8</td>
<td>99.82</td>
<td>100</td>
</tr>
<tr>
<td><em>pdk-1(sa709)</em></td>
<td>107†</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td><em>ced-9(n2812)</em></td>
<td>7.7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>ced-9(n2812); pdk-1(sa709)</em></td>
<td>0</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

† *pdk-1(sa709)* displays a serotonin-insensitive, partially penetrant *Egl* phenotype that results in the death of adults from internal hatching of progeny before the entire brood is laid.
NA = Not applicable
Worms were transferred to freshly seeded plates every 12 hours beginning at the L4 stage. Eggs laid were counted immediately after transfer and the percentage of these that hatched was determined 24 hours later. The number of F1 progeny reaching L4/Adulthood was counted when concurrently laid wild-type progeny reached this point.

*ced-4* likely functions downstream of *pdk-1* since the *ced-4(n1162)* null mutation was able to fully suppress the excessive apoptosis in *pdk-1(mg142)gf* mutants (Figure 3.4F). This argues that *pdk-1* functions through *ced-4* to regulate apoptosis, and that *daf-2* and *pdk-1* promote cell death from mutually independent pro-apoptotic pathways.

CED-4 translocates from mitochondria to the nuclear periphery during the course of developmental apoptosis in *C. elegans* (Chen et al., 2000; Conradt and Horvitz, 1998). Greiss and colleagues (2008) suggested that a similar phenomenon underlies DNA damage-induced germ cell apoptosis. I was interested in understanding whether *pdk-1* could control apoptosis through such a process, so I, in collaboration with Bin Yu, a technician in our lab, examined CED-4 localization and protein levels using α-CED-4 antibodies in germlines dissected from *pdk-1* mutants. Unlike Greiss *et al.* (2008), we did not detect any changes in CED-4 localization in response to IR in the wild-type and, in addition, we did not observe any changes in absolute protein levels (Figure 3.5). Furthermore, we found no difference between CED-4 levels or localization in wild-type versus *pdk-1* mutant germ cells (Figure 3.5). Therefore, it appears that *pdk-1* regulates CED-4 by a mechanism that does not include control of its subcellular localization or levels.
Figure 3.5 CED-4 localization does not change in response to IR or with respect to *pdk-1* status.

CED-4 localizes at the nuclear periphery (arrows) and this does not change with IR or in *pdk-1* mutants. Bright puncta in *pdk-1(gf)* mutant germ cells at 120 Gy were not reproducible in replicate experiments. CED-4 localization is not altered in actively dying cells (triangle), as determined by condensed chromatin in *pdk-1(gf)* mutants. In the terminal phases of cell death, CED-4 expression is lost in concert with the nuclear envelope (chevrons). Worms
were irradiated at the L4 stage and germlines were extruded after 24 hours at 25°C. Dissected germlines were fixed and stained with antibodies against CED-4 (Greiss et al., 2008) and the nuclear pore complex, as described in Material and Methods, followed by DNA staining with DAPI. Stained germlines were mounted on slides and visualized by epifluorescence microscopy. pdk-1(0) = pdk-1(sa680), pdk-1(gf) = pdk-1(mg142), ced-4(0) = ced-4(n2273) – a null allele (Yuan and Horvitz, 1992). Bar = 10 μm.

Partial reduction of germ cell apoptosis in ced-9(0) mutants caused by loss of pdk-1 (Figure 3.4E) suggested that pdk-1 could have a minor role in DNA damage-independent apoptosis. While there are minor defects in physiological apoptosis in the pdk-1(0) mutants, loss of pdk-1 function is not essential for this process to occur (Figure 3.2E). Neither does daf-2 appear to be essential for physiological apoptosis since its loss leads to a reduction in, but not a complete abrogation of, death in the absence of DNA damage (Figure 3.2B). Compared to ced-4 and ced-3, whose function is absolutely required for physiological apoptosis (Gumienny et al., 1999), daf-2 and pdk-1 play supporting roles in the regulation of damage-independent death. Because loss of Insulin-like signalling did not affect developmental apoptosis [5.3 ± 1.95 apoptotic cells in wild-type versus 6 ± 1.41 apoptotic cells in daf-2(e1370) 1.5-fold stage embryos (N = 10); Jordan Young, unpublished data], both daf-2 and pdk-1 appear to be major regulators of DNA damage-induced apoptosis that also have minor effects on damage-independent death in the C. elegans germline.

3.7 Parallel Signals from PDK-1/PDK1 and CEP-1/p53 are Required to Drive Damage-induced Germ Cell Apoptosis

cep-1 is required for damage-induced germ cell apoptosis (Derry et al., 2001; Schumacher et al., 2001). Recently, several regulators of IR-induced apoptosis [lin-35 (lin – abnormal cell LINeage) (Schertel and Conradt, 2007), sir-2.1 (sir – yeast SIRtuin related) (Greiss et al., 2008) and kri-1 (kri – human KRIIt1 homologue) (Ito et al., 2010)] have been demonstrated to promote death independently of cep-1. In these studies, it was not clear whether cep-1 function was required in parallel to lin-35, sir-2.1, and kri-1 or whether cep-1 was dispensible for apoptosis in these backgrounds; the availability of alleles that upregulate PDK-1 activity (Paradis et al., 1999) allowed me to answer this directly for pdk-1.
I reasoned that if *pdk-1* and *cep-1* were required in parallel to induce germ cell apoptosis, loss of *cep-1* might be able to suppress the sensitivity to apoptosis in *pdk-1(gf)* mutants, despite *pdk-1* being unable to regulate CEP-1 activity in response to DNA damage (Figure 3.4B). I generated a *cep-1(gk138); pdk-1(mg142)* double mutant and found that this was indeed the case (Figure 3.6). Furthermore, loss of the pro-apoptotic CEP-1 target gene *egl-1* also prevented apoptosis in *pdk-1(gf)* germ cells (Figure 3.6). In combination with earlier results suggesting that *cep-1* is not downstream of *pdk-1* (Figure 3.4), this suggested that CEP-1 transcriptional function is required in parallel to *pdk-1* to promote germ cell apoptosis. While these experiments cannot definitively rule out a transcription-independent role for CEP-1 (Green and Kroemer, 2009), they do demonstrate, for the first time, direct evidence of a simultaneous requirement for two pathways to control germ cell apoptosis in response to DNA damage. My data are also consistent with the existence of *cep-1* and *pdk-1*-independent pro-apoptotic pathways, as demonstrated by the small increases in apoptosis observed following IR in *cep-1; pdk-1* double mutants (Figure 3.6). Higher assay temperatures (25ºC in this case) are likely to have uncovered this. The simultaneous requirement for *cep-1* and *pdk-1* in the activation of damage–induced apoptosis has important implications for how cell death occurs *in vivo* and may help to explain why many different death-inducing pathways are targeted in cancer to evade apoptosis.

![Bar graph showing apoptosis rates](image)

**Figure 3.6 cep-1 and pdk-1 function in parallel to promote damage-induced germ cell apoptosis.**

L4 stage worms were treated with IR and germ cell apoptosis was quantified after 24 hours at 25ºC. *pdk-1(gf) = pdk-1(mg142), cep-1(lf) = cep-1(gk138), egl-1(lf) = egl-1(n1084n3082).* Error bars as in Figure 3.2.
3.8 AKT-1/Akt is Regulated by a Novel Pathway in Response to DNA Damage

The activity of Akt proteins is tightly controlled by phosphorylation, particularly on two conserved residues homologous to threonine 308 and serine 473 in human Akt1 (Alessi et al., 1996a) (Figure 1.8B). These phosphorylation events facilitate proper structural organization of the kinase domain for optimal substrate binding and catalysis (Yang et al., 2002a; Yang et al., 2002b). Because PDK-1 does not appear to function upstream of AKT-1 in response to DNA damage, I was interested in knowing whether other kinases could substitute for its function in vivo. Based on the published literature, I screened a list of kinases that could potentially regulate AKT-1 in a PDK-1-independent manner.

W04B5.5/PIAK (phospholipid-independent Akt kinase) encodes a C. elegans PDK-1-like kinase that lacks the carboxy-terminal pleckstrin-homology domain of PDK1 proteins. Previously, it was shown to regulate the phosphorylation and activity of human Akt in a heterologous transfection assay in human cells independently of PI3K (Li et al., 2001). When I examined two independent alleles of PIAK, ok1309 and tm2668, neither allele displayed sensitivity to IR-induced apoptosis in the germline, as would be expected from a positive regulator of akt-1 (Figure 3.7A). Because the two alleles also did not phenocopy each other, I attempted to validate my findings using RNAi. Unfortunately, RNAi against W04B5.5 was not penetrant (data not shown). While I cannot formally exclude PIAK as being upstream of AKT-1 in response to DNA damage, my current data do not support such a role. CKK-1 (Calcium-calmodulin Kinase Kinase) is the C. elegans homologue of the calcium and calmodulin-dependent protein kinase kinase (CaM-KK). CaM-KK can mediate activation of human Akt in response to calcium, probably by directly phosphorylating T308 (Yano et al., 1998). I tested the response of two alleles of ckk-1 to IR, but found that neither allele caused sensitivity (Figure 3.7B). Thus, ckk-1 does not appear to function simply upstream of akt-1 to promote its function.

Epidermal growth factor (EGF) signalling has an established role in DNA damage-dependent activation of Akt proteins through some PI3K-independent pathways (Contessa et al., 2002). To
investigate whether the EGF receptor (EGFR) could initiate a pathway that leads to the PDK-1-independent activation of AKT-1, I examined the response to IR of multiple let-23 alleles. LET-23 represents the single C. elegans homologue of EGFR (Manning, 2005) (LET – LEThal). Despite that fact that the C-terminal truncation allele sy1 demonstrated sensitivity to DNA damage, this was not phenocopied by additional alleles found 5’ to sy1 that are predicted to disrupt the carboxy terminus of the receptor (Sakai et al., 1996) (Figure 3.7C). Furthermore, loss of lin-7, which encodes a protein that is essential for determining the correct subcellular localization of LET-23 during vulval development, and which binds to LET-23 in the region deleted by the sy1 mutation (Kaech et al., 1998), did not lead to sensitivity to DNA damage-induced apoptosis (Figure 3.7C). This argues against a role for a LET-23-LIN-7-initiated pathway in the activation of AKT-1.

Figure 3.7 akt-1 is not regulated by kinases known to function upstream of Akt in mammals.
L4 stage (A, C) or young adult (B, D) worms were treated with IR and germ cell apoptosis was quantified after 24 hours at 20°C (B, C, D) or 25°C (A). Error bars in A as in Figure 3.2. Error bars in B-D represent the standard deviation from at least ten animals.

The mammalian target of rapamycin (mTOR) functions both upstream and downstream of Akt. As an upstream regulator, mTOR in its second complex, mTORC2, phosphorylates Akt at Ser473 (Hresko and Mueckler, 2005; Sarbassov et al., 2005), mediating an important step in PDK-1-dependent activation (Scheid et al., 2002). I examined null mutants in Rictor (rapamycin-insensitive companion of TOR), the mTOR binding partner that defines TORC2, but found that these mutants displayed severe defects in germline proliferation (unpublished observations) concomitant with reduced overall body size (Jones et al., 2009; Soukas et al., 2009). This hindered my further evaluation of TORC2 as an AKT-1 activator. Since there is no DNA-dependent protein kinase (DNA-PK) homologue in the worm (Manning, 2005), I can also exclude a pathway homologous to the DNA-PK-dependent activation of Akt1 described in mammals (Bozulic et al., 2008).

3.9 AKT-1/Akt May Function Independently of T350/T308 Phosphorylation

Because my data imply the existence of a novel pathway regulating AKT-1 in response to DNA damage, I wanted to understand the mechanism behind this regulation. Therefore, I raised antibodies against endogenous AKT-1 and used them to immunoprecipitate the protein from whole worms following IR. I verified that akt-1(ok525) is a protein null allele of akt-1 by Western blotting (Figure 3.8). Because the activity of Akt proteins is generally induced in response to extracellular stimuli (Manning and Cantley, 2007), I wanted to know whether a similar mechanism was used to control AKT-1 activity in response to DNA damage. This would also allow me to directly test my PDK-1-independent regulatory model for AKT-1 function. Although I, in collaboration with Bin Yu, could immunoprecipitate AKT-1 from whole worms, we were unable to detect kinase activity in two separate assays, one using the native substrate DAF-16 (Hertweck et al., 2004), and another based on a commercial kit which uses a GST-fused
glycogen synthase kinase (GSK)-3α Ser21 (Cross et al., 1995) peptide to quantify human Akt kinase activity non-radioactively (Cell Signalling Technology Akt Kinase Assay Kit; unpublished observations). While it is possible that the activity of AKT-1 is too insignificant to detect non-radioactively, it seems more likely that the conditions used during the lysis and purification of AKT-1 do not maintain the active state of the enzyme since purified human Akt functioned robustly in these assays (Bin Yu, personal communication). This is especially relevant when the difficulties inherent in lysing worms are considered: the cuticle is a robust structure that requires mechanical disruption by sonication or homogenization, coupled with detergent extraction to free soluble proteins. To accomplish this, harsh detergents such as sodium dodecylsulfate (SDS) are often used. However, even in the sparing amounts used in our protocol (0.1%), SDS probably still disrupts the activity of endogenous AKT-1. We attempted to substitute the non-ionic detergents nonyl phenoxypolyethoxylethanol (NP)-40 or Triton X-100 in the place of SDS, but were still unable to obtain active enzyme. Therefore, it is possible that the extraction conditions and/or substrates used in the current assays impede detection of active AKT-1. Future optimization would provide highly relevant insight into the DNA damage-dependent regulation of AKT-1.

Because my genetic data imply a model wherein AKT-1 functions independently of PDK-1, I wanted to know how the active state of AKT-1 was maintained in response to DNA damage. Phosphorylation at Thr308 is a key step in the control of Akt activity (Alessi et al., 1996a). Thus, my data could be accommodated by a model that includes an alternative kinase mediating Thr350 (the Thr308 homologue in AKT-1) phosphorylation (see above), or by a model that does not require Thr350 phosphorylation. To distinguish between these two possibilities, I used AKT-1 phospho-specific antibodies raised against the T350/T308 and Ser517 (the Ser473 homologue in AKT-1) phosphorylation sites (Padmanabhan et al., 2009) to examine AKT-1 phosphorylation status by Western blotting. In collaboration with Kelvin Yen and Heidi Tissenbaum (University of Massachusetts Medical School), we treated wild-type and akt-1(0) mutant worms with IR and examined T350/T308 and S517/S473 phosphorylation state by immunoblotting after immunoprecipitating total AKT-1 from worm lysates with my AKT-1 antibodies 24 hours after damage. Interestingly, T350/T308 and S517/S473 were both phosphorylated in the absence of damage, consistent with tonic activity models of Insulin-like signalling wherein the daf-2 pathway must be de-activated to allow entry into Dauer (Bargmann
and Horvitz, 1991; Li et al., 2003) (Figure 3.8). Surprisingly, however, IR did not alter phosphorylation at either residue over that seen in unirradiated controls. Because Akt activation in response to growth factors is transitory, phosphorylation at T308 is usually quite low in unstimulated tissues. It rises only after the introduction of growth factors and this closely parallels increases in Akt kinase activity (Alessi et al., 1996a). One would expect a similar pattern in response to IR, such that maximal AKT-1 T350 phosphorylation, and presumably kinase activity, would correlate with the time of maximal akt-1-dependent effects on apoptosis (24 hours after IR). Since mutation of T308 and S473 to non-phosphoacceptor residues (i.e. Ala) severely attenuates kinase activity in mammals (Alessi et al., 1996a), either phosphorylation at T350 and S517 is not important for DNA damage-dependent regulation of AKT-1, or AKT-1 operates in a non-canonical, perhaps even non-enzymatic way to control damage-induced apoptosis.

Figure 3.8 Phosphorylation of AKT-1 at Thr350 and Ser517 does not change in response to IR.

L4-stage worms were irradiated with the indicated dose and then incubated for 24 hours at 20°C. Worms were lysed and soluble AKT-1 was immunoprecipitated with a 1:1 mixture of AKT-1 antisera 128 and 527. Immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted to polyvinylidene fluoride (PVDF) before probing with phospho-specific antibodies against T350 or S517 of AKT-1 (Padmanabhan et al., 2009). Blots were then stripped and reprobed with a 1:1 mixture of total AKT-1 antibodies 128 and 527. akt-1(0) = akt-1(ok525).
To gain further insight into the mechanisms regulating AKT-1, we further examined the phosphorylation of T350 and S517 in *pdk-1* and *daf-2* mutants in response to IR. If AKT-1 truly functions independently of these PI3K signalling components, it is possible that T350 phosphorylation is maintained by another, as yet unidentified kinase, or that phosphorylation at these sites does not play a major role in regulating IR-specific AKT-1 activity. In accord with Figure 3.8, we did not observe large changes in T350 phosphorylation in wild-type worms after 120Gy of IR (Figure 3.9A). This was confirmed by directly quantifying T350 phosphorylation levels by densitometry (Figure 3.9B). Interestingly, densitometry did reveal a near doubling in S517 phosphorylation after DNA damage, suggesting that perhaps AKT-1 is regulated mainly at this site (Figure 3.9B). Because this data suggests that changes in T350 phosphorylation status may not be important for the DNA damage-dependent regulation of AKT-1, we wanted to exclude the possibility that a *pdk-1*-independent kinase was mediating T350 phosphorylation in response to IR. Such a model would suggest that PDK-1 maintains the phosphorylation of T350 in the absence of damage and that upon IR, PDK-1 activity would be swapped with that of another kinase that maintains phospho-T350 levels. The net result of such a scenario would be the same as if changes in T350 phosphorylation did not affect regulation of AKT-1.

Interestingly, when PDK-1 activity was reduced by the *sa709* hypomorphic mutation (Paradis et al., 1999), phospho-T350 levels were only marginally reduced both before and after IR (Figure 3.9). Because phospho-T530 levels were not completely ablated by the *sa709* mutation [as would be expected for total loss of PDK-1 activity (Alessi et al., 1997a)], we attempted to further deplete *pdk-1* activity by using the *sa680* putative null mutation. Unfortunately, we were unable to isolate sufficient numbers of adult *pdk-1(sa680)* worms from which to extract protein for AKT-1 immunoprecipitations (Kelvin Yen, unpublished observations). Because our phospho-specific antibodies are only able to detect phosphorylated AKT-1 after immunoprecipitation (Padmanabhan et al., 2009), and since *pdk-1* RNAi is not penetrant by feeding [ (Paradis et al., 1999) and unpublished observations], we sought to corroborate our results using the *pdk-1*(*mg142*) gf mutation. Surprisingly, upregulation of *pdk-1* activity also did not significantly affect T350 phosphorylation and may have even reduced it (Figure 3.9B). This argues against a role for PDK-1 in maintaining AKT-1 T350 phosphorylation both in the absence and presence of DNA damage, and instead suggests that another kinase could mediate both processes. While these data require replication, they presently conflict with previously published models of *C. elegans* PI3K signalling that have invoked a direct phosphorylation model to explain the
structure of the \textit{pdk-1-akt-1} cassette (Gami et al., 2006; Hertweck et al., 2004; Paradis et al., 1999). Since strong genetic evidence exists to place \textit{pdk-1} upstream of \textit{akt-1} in the regulation of dauer arrest (Paradis et al., 1999), it is possible that a direct phosphorylation model does not suffice. Perhaps phosphorylation-independent mechanisms, such as a direct interaction between PDK-1 and AKT-1, can account for the genetic data linking \textit{pdk-1} and \textit{akt-1}. In this light, no biochemical verification of direct AKT-1 phosphorylation by PDK-1 has been presented in the \textit{C. elegans} literature as yet. In the future, it will be important to verify a direct phosphorylation model in dauer arrest and to determine whether AKT-1 is still catalytically active in the germline in the absence of \textit{pdk-1}.
Figure 3.9 *daf-2* and *pdk-1* differentially regulate Thr350 and Ser517 phosphorylation status.

A) Worms were irradiated and protein immunoprecipitated as described in Figure 3.8. All samples were run on the same gel, transferred to PVDF and probed for T350. Secondary antibodies conjugated to horseradish peroxidase were inactivated with 0.1N HCl and then blots were probed for total AKT-1 using a 1:1 mixture of AKT-1 antisera 128 and 527. To detect S517, blots were stripped and re-probed for with S517 antibodies as described in Figure 3.8. Bands were separated during figure preparation to ease visualization. *pdk-1(lf) = pdk-1(sa709); pdk-1(gf) = pdk-1(mg142); daf-2(lf) = daf-2(e1370).*  

B) The intensity of T350 and S517 in each lane was quantified using ImageJ.
and normalized to AKT-1. The normalized intensity of each band is expressed relative to wild-type unirradiated.

Data represent the average of 1-2 experiments.

Although T350 phosphorylation was not grossly affected by \textit{pdk-1} status, we did notice a significant (~ 3-fold) upregulation of S517 phosphorylation upon loss of \textit{pdk-1} in the absence and presence of IR (Figure 3.9A). This could reflect the relief of a negative feedback loop such as that described in mammals - excessive Akt-induced TORC1 activity leads to phosphorylation of IRS1 and the downregulation of Akt-inducing PI3K activity (Harrington et al., 2005). Such a pathway was demonstrated \textit{in vivo} in studies of \textit{Drosophila} (Radimerski et al., 2002). Specifically, knockdown of \textit{Drosophila PDK1} (\textit{dPDK1}) decreased TORC1 feedback signalling to dInsR and dIRS1 [Chico; (Kockel et al., 2010)] and thereby increased dAkt S473 phosphorylation by presumed upregulation of TORC2 S473-directed kinase activity (Yang et al., 2006). Relief of a similar \textit{pdk-1}-dependent feedback loop in \textit{C. elegans} could lead to a similar upregulation of S517 phosphorylation by TORC2. However, it is likely that such a feedback loop does not take the form of the IRS1-based loop outlined above because knockdown of \textit{ist-1}, the \textit{C. elegans IRS1} homologue, had no effect on reproduction nor damage-induced germ cell apoptosis (Figure 3.7D). Furthermore, the \textit{pdk-1(mg142)}gf mutation was unable to revert the excessive phosphorylation of S517, which would be expected if a \textit{pdk-1}-dependent feedback mechanism existed to control AKT-1 phosphorylation (Figure 3.9B). It will be important to further evaluate the validity of a feedback mechanism through S517 by examining mutants that reduce \textit{pdk-1} activity below that in \textit{pdk-1(sa709)}. If such a feedback loop does exist in \textit{C. elegans}, it is likely to function in parallel to damage-induced S517 phosphorylation since the loss of \textit{pdk-1} activity and IR seem to have additive effects on S517 phosphorylation (Figure 3.9B).

Most surprisingly, loss of \textit{daf-2} completely abrogated phosphorylation of both T350 and S517 in the unirradiated state (Figure 3.9A). This argues that \textit{daf-2} activity is essential for maintenance of basal AKT-1 phosphorylation and that perhaps \textit{daf-2} can maintain AKT-1 T350 and S517 phosphorylation independently of \textit{pdk-1}. This argues against current models of the \textit{C. elegans} PI3K pathway (Gami et al., 2006; Hertweck et al., 2004; Paradis et al., 1999). If one presumes that AKT-1 is active in the germline irrespective of \textit{daf-2} status, the failure of IR to restore T350 phosphorylation in \textit{daf-2} mutants strongly suggests that T350 does not require modification to be functional in response to IR. Obviously, this would have to be validated by directly assessing
AKT-1 kinase activity, but the failure to restore T350 and S517 phosphorylation with IR in *daf-2(1f)* provides compelling evidence that stimulus-specific functions of AKT-1 could be controlled by differential phosphorylation: T350 in the regulation of dauer arrest; and no phosphorylation, or perhaps phosphorylation only at S517 in the control of damage-induced germ cell apoptosis. The robust increase in T350 phosphorylation (~2-fold) seen in fed worms over that in their starved counterparts supports this working model (Figure 3.9A).

The differential effects of *daf-2* and *pdk-1* on AKT-1 phosphorylation support my genetic data that separates these two PI3K components from *akt-1*. Furthermore, the inability of *pdk-1* to significantly regulate the phosphorylation of T350 in AKT-1, a site key to the regulation of Akt by PDK1 proteins in mammals (Alessi et al., 1997a), and the presumed ability of AKT-1 to function independently of *daf-2*-dependent phosphorylation events, suggests the existence of a novel DNA damage-dependent regulatory mechanism for AKT-1. Since recruitment to the membrane and subsequent phosphorylation by PDK1 is a key step controlling the PI3K-dependent activation of Akt (Andjelkovic et al., 1997; Bellacosa et al., 1998), I wanted to further assess whether this potentially novel AKT-1 regulatory mechanism involved DNA damage-dependent alterations in AKT-1 subcellular localization. To this end, we dissected germlines from worms treated with IR and stained them with AKT-1 antibodies. Unfortunately, and despite significant optimization with the protein null *akt-1(ok525)* allele (see above), Bin Yu and I were unable to obtain specific staining of AKT-1 in the germline (data not shown). To rule out deficiencies in our protocol, we tested whether we could detect AKT-1 over-expressed as a transgene linked to GFP in the soma (Padmanabhan et al., 2009). Indeed, we were able to recapitulate the reported expression pattern in the anterior nerve ring, the ventral cord and the spermatheca (Paradis and Ruvkun, 1998), which was verified by signal from the GFP tag (unpublished observations). When we repeated this experiment on wild-type worms that express only endogenous AKT-1, however, we could not detect any staining at these sites or anywhere else. This suggested that my antibodies are of only sufficient affinity to detect large amounts of readily-accessible AKT-1. We did notice that somatic AKT-1::GFP does not change localization in response to DNA damage (unpublished observations).

To investigate whether staining of somatic AKT-1 could provide insight into the damage-dependent regulation of this kinase, we examined the tissue expression requirements for *akt-1* in
response to IR. *rrf-1* (*rrf* – Rna-dependent Rna polymerase Family) encodes an RNA-dependent RNA polymerase required for RNAi in the soma (Sijen et al., 2001). Conversely, *ppw-1* (*ppw* – Paz/PiWi domain-containing) encodes a PiWi domain protein required for RNAi in the germline (Tijsterman et al., 2002). Using RNAi knock down of *akt-1* in either *rrf-1* or *ppw-1* mutants, I assessed from which location *akt-1* expression was required to regulate germ cell apoptosis. *akt-1* RNAi in the wild-type phenocopied the sensitivity to damage-induced apoptosis caused by *akt-1*(0) mutations and the effect of *akt-1* RNAi was similar in *rrf-1*(0) mutants (Figure 3.10A). Conversely, *akt-1* RNAi did not sensitize *ppw-1*(lf) mutant germ cells to DNA damage (Figure 3.10A). Since the effects of *akt-1* RNAi were maintained in *rrf-1*(0) mutants, which are competent for germline RNAi (Sijen et al., 2001), and was lost in *ppw-1*(lf) mutants, which have defects in germline RNAi (Tijsterman et al., 2002), *akt-1* expression seems to be required cell-autonomously in the germline to inhibit damage-induced apoptosis. To confirm the germline expression requirement for *akt-1* suggested by these tissue-specific RNAi experiments, I examined whether a high-copy extrachromosomal array expressing *akt-1* could revert the sensitivity to IR-induced germ cell apoptosis caused by *akt-1*(0) mutations. Gene expression from high-copy arrays is generally silenced in the germline (Kelly and Fire, 1998; Kelly et al., 1997). In line with my *akt-1* RNAi results in *ppw-1* mutants (Figure 3.10A), the presence of the high copy *byEx[akt-1(+)]* array in *akt-1*(ok525) mutants was unable to revert sensitivity to DNA damage-induced germ cell apoptosis (Figure 3.10B). Because *byEx[akt-1(+)]* efficiently rescues the somatic phenotypes of *akt-1*(ok525) (Hertweck et al., 2004), this experiment strongly suggests that *akt-1* functions in the germline to regulate apoptosis. Therefore, in the absence of specific germline staining of AKT-1, I cannot further evaluate how changes in the subcellular localization of germline AKT-1 may contribute to its regulation in response to DNA damage.
Figure 3.10 *akt-1* functions in the germline to control damage-induced germ cell apoptosis.

**A)** Young adult worms (raised from the L1 stage on *akt-1* or control RNAi) were transferred to fresh RNAi plates, treated with IR, and germ cell apoptosis was quantified after 24 hours at 20°C. Error bars as in Figure 3.2. *rrf-1(0) = rrf-1(pk1417), ppw-1(lf) = ppw-1(pk1425). * = p < 0.05 versus control(RNAi).*  

**B)** Young adult worms were treated with IR and germ cell apoptosis was quantified after 24 hours at 20°C. *byEx[akt-1(+)]* is a high-copy extrachromosomal array that rescues the somatic Daf-c phenotype of *akt-1(ok525); akt-2(RNAi)* worms (Hertweck et al., 2004). Error bars are as in Figure 3.2. *akt-1(0) = akt-1(ok525). * = p < 0.05 versus wild-type.*
4 Results 2: AKT-1/Akt Targets ATL-1/ATR to Dampen Pro-apoptotic Signalling

4.1 Data Attribution

All data presented in this chapter were acquired solely by the author.

4.2 AKT-1/Akt Regulates CEP-1/p53 in an Mdm2-independent Manner

AKT-1 can regulate the function of CEP-1 by dampening its phosphorylation in response to DNA damage (Quevedo et al., 2007). Since phosphorylation of p53 proteins is important for their activation (Lavin and Gueven, 2006), this represents a powerful way to control the function of CEP-1. The exact mechanism(s) by which AKT-1 achieves this is unknown. In mammals, growth factors are able to regulate the function of p53 through a pathway that involves Akt-dependent phosphorylation of the E3 ubiquitin ligase Mdm2. This phosphorylation induces a shift of Mdm2 from the cytoplasm to the nucleus (Mayo and Donner, 2001; Zhou et al., 2001), where it is able to bind to p53 and induce its degradation (Feng et al., 2004; Gottlieb et al., 2002; Ogawara et al., 2002). Although the effects of DNA damage on this relationship were not explored in human cells, it remained possible that a homologous, DNA damage-dependent pathway existed in the worm to regulate CEP-1 protein levels, perhaps even the phosphorylated form of CEP-1 specifically. When knockdown of none of the worm E3 ubiquitin ligases that contained Akt consensus phosphorylation sites was able to revert the resistance of akt-1(gf) mutants to damage-induced apoptosis (Quevedo et al., 2007), I hypothesized that perhaps AKT-1 could regulate CEP-1 independently of Mdm2-like ligases. This is entirely consistent with the lack of an identifiable mdm2 homologue in the C. elegans genome (Lane et al., 2010).
4.3 The DNA Damage-responsive Kinases ATL-1/ATR and ATM-1/ATM Contain Putative Akt Consensus Phosphorylation Sites

Because the phosphorylation state of CEP-1 is controlled by DNA damage-responsive kinases, I reasoned that instead of targeting an Mdm2-like protein, Akt could inhibit a kinase that phosphorylates CEP-1. Four key DNA damage-responsive kinases are thought to target p53 proteins, including ATR, ATM, and the checkpoint kinases Chk1 and Chk2. The *C. elegans* homologue of Chk2, CHK-2, does not regulate IR-induced apoptosis (MacQueen and Villeneuve, 2001; Stergiou et al., 2007), and thus, I could immediately exclude it as a potential AKT-1 target. To further narrow my search, I took advantage of the peptide motif scanning algorithm Scansite. This algorithm can detect potential phosphorylation sites in a given protein by searching for short sequence motifs that match the empirically-determined consensus phosphorylation site for a given kinase (Obenauer et al., 2003). The *in silico* power of Scansite is underlaid by actual data from phage display or *in vitro* kinase assays, and information from validated *in vivo* phosphorylation sites, when available. This allows results to be ranked against the performance of validated sites in the same algorithm (Figure 4.1A). When I scanned the primary sequences of the ATM, ATR, and Chk1 orthologues from worms, I was surprised to observe that *C. elegans* Chk1/CHK-1 did not contain any predicted consensus phosphorylation sites. This is despite the existence of a mammalian pathway wherein Akt directly phosphorylates Chk1 to bypass the G2/M checkpoint (King et al., 2004; Puc et al., 2005; Shtivelman et al., 2002). Thus, AKT-1 may target a novel kinase to dampen signalling to CEP-1. In line with this, I detected 17 putative sites in a low-stringency screen of the ATR homologue ATL-1 (Figure 4.1B) and 4 putative sites in the ATM homologue ATM-1 (Figure 4.1C). The sites detected in ATM-1 were of noticeably poor predictive value when compared to those in ATL-1 [the closest to the consensus site of RXRXXS/T (Alessi et al., 1996b; Obata et al., 2000) being 474-RQSLAT-481 in ATM-1]. On the contrary, the best site in ATL-1 (477-RDRVGS-484) closely resembled the consensus and may be conserved in mammalian ATR (430-RRRLSS-437; Figure 4.1B). This suggested that either kinase could serve as a target of AKT-1, with ATL representing the more likely; however, *in silico* prediction of protein phosphorylation sites is not definitive, thus I proceeded to test these predictions *in vivo.*
Figure 4.1 ATL-1 contains putative Akt consensus phosphorylation sites, as detected by Scansite.

A) Scansite compares a putative Akt consensus phosphorylation site against a library of validated sites from in vitro and in vivo studies (Obenauer et al., 2003). It then ranks this site to provide a predictive measure of putative site quality.  

B) ATL-1 contains many predicted Akt consensus phosphorylation sites. The highest ranking site, Ser483, is expanded. A similar site is found in a homologous position in ATR (below). Other sites in ATL-1, ranked as follows, are: T656, S1356, S1970, S516, T849, T1432, T1053, T509, S1765, S519, T2257. Residue identity between the different functional domains of ATL-1 and ATR are given (Garcia-Muse and Boulton, 2005).  

C) Predicted Akt consensus sites in ATM-1. While the atm-1 gene is annotated to encode a protein of only 649 amino acids, other ATM proteins usually comprise upwards of 1500-2500 amino acids. Scansite predicts four putative Akt sites in ATM-1, all of poor quality when compared to those detected in ATL-1. Putative Akt sites in ATM-1 are ranked as follows: T480, S124, S62, S496.
4.4 *atl-1/ATR* Functions Downstream of *akt-1/Akt* in Response to DNA Damage

If AKT-1 targets either ATL-1 or ATM-1, the hypersensitivity of *akt-1(0)* mutants to DNA damage-induced apoptosis should be suppressed by simultaneous loss of either kinase. Garcia-Muse and Boulton (2005) reported that *atm-1(RNAi)* worms were completely resistant to IR-induced apoptosis at doses up to and including 75 Gy. Using the predicted null mutation *gk186*, Stergiou and colleagues (2007) found that *atm-1* was only required for germ cell apoptosis at low doses of IR (*i.e.* 15 and 30 Gy); *atm-1(gk186)* displayed wild-type levels of germ cell apoptosis at doses higher than 60 Gy. Since these two studies conflicted, I first tested whether loss of *atm-1* caused resistance to IR-induced germ cell apoptosis. I found that *atm-1* RNAi was not penetrant (unpublished observations), so I instead examined the response to *atm-1(gk186)* mutants to IR. When compared to wild-type I did not observe resistance to germ cell apoptosis in *atm-1(gk186)* worms at 15, 30, or 60 Gy, unlike Stergiou and colleagues (2007) (Figure 4.2). Because I could not replicate IR-specific results in *atm-1(gk186)* mutants, I further tested whether I could observe resistance of *atm-1(gk186)* mutants to ultraviolet (UV) radiation-induced germ cell apoptosis. UV can elicit *cep-1*-dependent germ cell apoptosis (Derry et al., 2007; Stergiou et al., 2007) and *atm-1* is required for this apoptosis at both low and high doses of UV (*e.g.* 15-100 J/m²) (Stergiou et al., 2007). In response to UV, *atm-1(gk186)* did cause resistance to apoptosis, but this did not suppress the hypersensitivity of *akt-1(0)* to UV (Figure 4.3). This suggested that *atm-1* does not function downstream of *akt-1* in response to UV; however, because of the inconsistencies with published literature that I observed with *atm-1(gk186)* in response to IR, I could not fully evaluate the role of ATM-1 as an AKT-1 target. It is possible that *akt-1* functions independently of *atm-1* since *atm-1(gk186)* causes synthetic lethality with mutations in *hpr-9*, *mrt-2*, and *hus-1* (Stergiou et al., 2007) while *akt-1(ok525)* does not (Quevedo et al., 2007).
Figure 4.2 The *gk186* allele of *atm-1* does not cause resistance to IR-induced apoptosis.

Young adult worms were irradiated with the indicated dose of IR and apoptosis was quantified after 24 hours incubation at 20°C. Error bars represent the standard deviation of the mean from at least ten animals; only one gonad arm was examined in each animal.

Figure 4.3 *atm-1* does not function downstream of *akt-1* in response to ultraviolet (UV) radiation.

Young adult worms were irradiated with 100 J/m² of UV in a UV cross-linker and apoptosis was quantified after 24 hours incubation at 20°C. Error bars are as in Figure 4.2.

*atl-1* encodes the DNA damage-responsive kinase most responsible for the transmission of signals that promote *cep-1*-dependent germ cell apoptosis (Aoki et al., 2000). Thus, IR-induced germ cell apoptosis has a strict requirement for *atl-1* (Garcia-Muse and Boulton, 2005; Stergiou et al., 2007). In unicellular organisms such as yeast, ATR-like proteins also represent the major
means of transmitting DNA damage-induced signals (Morrow et al., 1995). The existence of many predicted Akt phosphorylation sites in ATL-1 and their potential conservation in the human homologue ATR suggested that ATL-1 could represent a good target through which AKT-1 could dampen apoptotic signalling in the germline. Consistent with this, loss of atl-1 in the tm853 allele, which is a protein null (Garcia-Muse and Boulton, 2005), suppressed the hypersensitivity of akt-1(0) mutant germ cells to IR at all doses tested (Figure 4.4A). This strongly suggests that atl-1 functions downstream of akt-1 in response to DNA damage. To further characterize this epistasis, I irradiated worms and followed the timecourse of apoptosis in atl-1(tm853)akt-1(RNAi) double mutants. Within six hours of irradiation, apoptosis was induced in wild-type worms (Figure 4.4B). Interestingly, the hypersensitivity of akt-1(RNAi) worms to IR did not become apparent until 6 hours later (12 hours post-IR). This suggested that AKT-1 activity may be induced after DNA damage, rather than being constantly maintained in the germline. In the context of my data demonstrating that a novel pathway regulates akt-1 in response to DNA damage (see Results 1), this argues for an inductive process that is specifically activated by IR. At later time points, the akt-1(RNAi) phenotype was manifest, and atl-1(tm853) was able to fully suppress this (Figure 4.4B). This strongly argues that atl-1 functions downstream or independently of akt-1 in response to DNA damage. It is unlikely that the suppression observed is the result of delayed apoptosis in atl-1(tm853) since germ cell death in these mutants was absent at all timepoints up to 24 hours (Figure 4.4B). Ancillary observation of my dose response and time course analysis indicated that there was a small, statistically insignificant rise in germ cell apoptosis in atl-1 and atl-1akt-1(RNAi) double mutants after IR, when compared to unirradiated controls. Although this represents an increase of only ~2-3 dying cells, it may reflect the minor contribution of an atl-1-independent pro-apoptotic pathway.
Figure 4.4 Loss of atl-1 suppresses DNA damage-induced germ cell apoptosis in akt-1(RNAi) worms.

A) Dose response analysis. Young adult worms were irradiated with the indicated dose of IR and apoptosis was quantified after 24 hours incubation at 20°C. atl-1(0) = atl-1(tm853). Error bars represent the standard error of the mean (SEM) from at least three independent experiments. At least ten animals were examined in each experiment.

B) Kinetics of suppression. Young adult worms were irradiated with 120 Gy (left) or left untreated (right) and germ cell apoptosis was quantified 6, 12, and 24 hours later. At times in excess of 24 hours, significant degeneration of akt-1(RNAi) germlines occurred, precluding further analysis. Error bars are as in A.

Because akt-1 and akt-2 function in independent genetic pathways to regulate damage-induced germ cell apoptosis (Quevedo et al., 2007), I wanted to understand how akt-2 functioned in relation to atl-1. Unlike in the case of akt-1(RNAi), loss of atl-1 in akt-2(ok393) null mutants (Hertweck et al., 2004) only partially suppressed IR-induced germ cell apoptosis (Figure 4.5). This suggested that atl-1 does not function directly downstream of akt-2 and that akt-2 may instead function independently of atl-1. This supports previous data demonstrating that akt-1
and *akt-2* function in independent genetic pathways to regulate damage-induced germ cell apoptosis (Quevedo et al., 2007).

Figure 4.5 *akt-2* functions independently of *atl-1* to regulate DNA damage-induced germ cell apoptosis.

Young adult worms were treated as in Figure 4.4. *atl-1(0) = atl-1(tm853), akt-2(0) = akt-2(ok393).* Error bars are as in Figure 4.4.

### 4.5 AKT-1/Akt May Regulate Multiple Aspects of ATL-1/ATR Function

The presence of putative Akt consensus phosphorylation sites in ATL-1 suggested that AKT-1 could directly target ATL-1 to dampen its function. As part of their activation process, ATR proteins, including ATL-1, are recruited to sites of DNA damage (Garcia-Muse and Boulton, 2005; Tibbetts et al., 2000), placing them close to binding partners that induce kinase activation (Bonilla et al., 2008). These two steps of ATL-1 activation - recruitment to sites of DNA damage and activation by binding partners – represent likely points at which AKT-1 could intervene in pro-apoptotic signalling.

As polymerases replicating DNA encounter damage, their progress along the template strand can become stalled in relation to other replication complex components. This can cause significant regions of single-stranded DNA (ssDNA) to become exposed as errant helicases continue to unwind template DNA (Byun et al., 2005); these tracts of ssDNA represent powerful targets for
the activation of ATR (You et al., 2002) (Figure 4.6, left). Naked ssDNA, being relatively labile to nucleases, is rapidly covered by the tri-partite replication protein A (Mordes and Cortez, 2008) (RPA) and it is this RPA bound to ssDNA that recruits ATR (Ball et al., 2007; Dubrana et al., 2007; Zou and Elledge, 2003) through its obligate binding partner ATRIP (ATR interacting protein) (Cortez et al., 2001; Edwards et al., 1999). RPA-ssDNA complexes also recruit other initiators of DNA damage signalling, including the 9-1-1 complex homologous to the worm HPR-9-MRT-2-HUS-1 checkpoint clamp. 9-1-1 recruitment to sites of RPA-ssDNA is a complex process that occurs independently of ATR-ATRIP binding to RPA (Kondo et al., 2001; Melo et al., 2001; Zou et al., 2002). It involves the loading of the circular 9-1-1 clamp (Dore et al., 2009) onto DNA by the Rad17-specific loader (Zou et al., 2002) at sites where a 5' end of double-stranded DNA (dsDNA) adjoins ssDNA (Ellison and Stillman, 2003). Loading of 9-1-1 at 5' DNA junctions generates a powerful ATR-activating structure (MacDougall et al., 2007), but free 5' DNA ends are not normally present during DNA replication since lagging strand synthesis rapidly removes them by primer resection, gap filling and subsequent ligation. In contrast, DNA damage-dependent helicase uncoupling generates large stretches of ssDNA that are substrates for polymerase-α (pol-α), a DNA polymerase that synthesizes DNA in the absence of a primed site (Henner and Further, 1977). Pol-α thus generates de novo 5' ends for 9-1-1 complex recruitment (Byun et al., 2005; Yan and Michael, 2009), through a direct RAD9-RPA interaction (Majka et al., 2006a; Xu et al., 2008; Zou et al., 2003). This exposure of ssDNA, its subsequent priming by pol-α, and 9-1-1 loading at free 5' ends has been proposed as a common pathway for ATR activation (MacDougall et al., 2007) (Figure 4.6, bottom).

Additional studies have shown that ATR activation can occur outside of the context of stalled replication forks (Tibbetts et al., 1999). In this scenario, the common ATR activating structure of a 5' primer-template junction is formed after MRE11-RAD50-NBS1 (MRN) complex-directed processing of DNA double strand breaks (Adams et al., 2006; Jazayeri et al., 2006; Myers and Cortez, 2006; Nakada et al., 2004) (Figure 4.6, right). The free ssDNA can then be bound by RPA and presumably recruit 9-1-1 to pol-α-generated 5' ends. Since mre-11 is required for the recruitment of ATL-1 to sites of IR-induced damage in the C. elegans germline (Garcia-Muse and Boulton, 2005), it seems that this mode of ATR activation is conserved in the worm.
By assembling near each other on contiguous regions of RPA-primed ssDNA, ATR and the 9-1-1 complex can follow a common pathway for ATR activation by forming a tripartite complex with TopBP1 (Topoisomerase II Binding Protein 1) (Delacroix et al., 2007; Lee et al., 2007) (Figure 4.6, bottom). Strong potentiation of basal ATR activity (Kumagai et al., 2006) is accomplished by TopBP1 bridging the 9-1-1/ATR-ATRIP complex through direct interactions with the phosphorylated C-terminus of RAD9 (Delacroix et al., 2007; Lee et al., 2007) and ATR-ATRIP (Mordes et al., 2008a). This is presumed to relay the presence of damaged DNA to ATR and to translate this into structural re-arrangements in the kinase domain that facilitate activation (Mordes and Cortez, 2008). In the absence of a detailed three-dimensional understanding of ATR regulation, the mechanistic underpinnings of this can only be hypothesized, but they probably involve re-orientation of catalytic and substrate-binding residues to facilitate optimal phosphotransfer.
Figure 4.6 Single-stranded DNA serves as a common substrate for ATR activation.

Induced lesions can stall polymerases and uncouple helicase activity from replicative procession (Byun et al., 2005). This exposes large stretches of single-stranded DNA (ssDNA; upper left). ssDNA can also be generated by MRN-directed (Buis et al., 2008) nuclease resection at sites of DNA double strand breaks (upper right). The resultant
ssDNA stretches are primed by DNA polymerase-α (pol-α) (Lupardus et al., 2002; Yan and Michael, 2009) to generate free 5' end junctions that specify RAD9-RAD1-HUS1 (9-1-1) complex loading (Ellison and Stillman, 2003; Majka et al., 2006a) by the RAD17-specific loader (Zou et al., 2002; Zou et al., 2003). The tripartite replication protein A (RPA) binds to naked ssDNA, promoting 9-1-1 complex loading (Zou et al., 2003) and facilitating recruitment of ATR-ATRIP (Zou and Elledge, 2003). By bridging the 9-1-1 complex and ATR-ATRIP (Delacroix et al., 2007; Lee et al., 2007; Mordes et al., 2008a) Topoisomerase II Binding Protein 1 (TopBP1) facilitates ATR activation and maximal kinase activity (Kumagai et al., 2006).

I hypothesized that the activation of ATL-1 was dependent upon a similar string of events in C. elegans since the TopBP1-dependent activation of ATR proteins is conserved from yeast to humans (Mordes et al., 2008b; Navadgi-Patil and Burgers, 2008; Puddu et al., 2008). Because both hpr-9-mrt-2-hus-1 and atl-1 modulate CEP-1 activity and DNA damage-induced apoptosis (Garcia-Muse and Boulton, 2005; Hofmann et al., 2002), I wondered whether the worm TopBP1 homologue mus-101 (Holway et al., 2005) was also required for these processes (mus – MUtagen Sensitive). mus-101 has essential functions in the initiation of DNA replication as well as during DNA damage signalling. Therefore, I found it necessary to partially deplete mus-101 levels by feeding RNAi, as described (Holway et al., 2005). This level of knockdown was sufficient to completely abrogate the pro-apoptotic functions of mus-101 (Figure 4.7) but not severe enough to cause the widespread embryonic death caused by its complete absence (Holway et al., 2005). Interestingly, loss of akt-1 was only partially suppressed by knockdown of mus-101, suggesting that the two genes function in independent pathways (Figure 4.7). A similar partial suppression of akt-1(0) was observed in mrt-2; akt-1 and hus-1; akt-1 double mutants (Quevedo et al., 2007). Because the 9-1-1 complex can activate ATR independently of TopBP1 in vitro (Majka et al., 2006b), it is possible that mus-101 and hpr-9-mrt-2-hus-1 could co-operatively determine ATL-1 activation in vivo.
Figure 4.7 AKT-1 could target multiple aspects of ATL-1 regulation.

Worms were raised from the L1 stage on *mus-101* or control RNAi, irradiated with the indicated dose as young adults, and then apoptosis was quantified after 24 hours at 20°C. Error bars are as in Figure 4.4A.

Partial suppression of *akt-1(0)* by loss of 9-1-1 components or *mus-101* could also suggest that AKT-1 targets multiple aspects of ATL-1 regulation. Interestingly, mammalian Akt can promote TopBP1 oligomerization by direct phosphorylation. This allows oligo-TopBP1 to bind E2F1 and prevent apoptosis (Liu et al., 2006). Ser1159, the site of Akt phosphorylation in human TopBP1, is conserved in mice and frogs (Liu et al., 2006), but does not have an easily identifiable homologue in MUS-101 (Figure 4.8). The shorter length of MUS-101 compared to TopBP1 homologues could hide a cryptic Ser1159-like site or AKT-1 could regulate MUS-101 independently of phosphorylation at a Ser1159-like site. Further insight into the mechanism of MUS-101 regulation by AKT-1 awaits the analysis of *hus-1* *mus-101(RNAi); akt-1* and *mus-101(RNAi); mrt-2; akt-1* triple mutants. If 9-1-1 and MUS-101 do indeed function in parallel to promote ATL-1 activation, damage-induced apoptosis should be completely abrogated in these compound mutants. In addition, the presence of potential direct phosphorylation sites in ATL-1 suggests that AKT-1 could regulate the ATL-1 polypeptide independently of 9-1-1 or MUS-101. Perhaps AKT-1 is able to control both the recruitment of ATL-1 to sites of DNA damage by direct phosphorylation, and its subsequent activation by 9-1-1-MUS-101. I am currently examining this possibility using antibodies to detect ATL-1 recruitment to sites of damage in *akt-1* mutants. If *akt-1* can control both aspects of ATL-1 function, this would represent a powerful mechanism with which to dampen damage-induced germ cell apoptosis.
Figure 4.8 Ser1159, a site of Akt phosphorylation in human TopBP1, may not be conserved in MUS-101.

Ser1159 in human TopBP1 is directly phosphorylated by Akt (arrow) (Liu et al., 2006). Ser1159 resides between the 6th and 7th BRCT (breast cancer susceptibility protein C-terminal domain) domains of TopBP1 and is conserved in frogs, mice, and rats (not shown) (Liu et al., 2006). MUS-101 is the *C. elegans* TopBP1 homologue (Holway et al., 2005), although it is shorter than mammalian orthologues and only contains 6 BRCT domains instead of 8. Block Maker (Henikoff et al., 1995) was used to construct the best alignment from full-length input sequences.
5 Conclusion

5.1 DNA Damage-dependent Re-arrangements of the Phosphatidylinositol 3-kinase Signalling Pathway

Given the conserved role of the PI3K pathway in preventing apoptosis (Liu et al., 2009; Vivanco and Sawyers, 2002), the results of this study are surprising. They indicate that signalling from the RTK DAF-2 and PDK1 homologue PDK-1 are required to promote cell death, completely opposite to the situation seen in mammals. This is also at odds with previous studies which classified daf-2 as having an anti-apoptotic role in damage-induced germ cell apoptosis (Pinkston-Gosse and Kenyon, 2007; Pinkston et al., 2006). The strong pro-apoptotic role observed in my study was confirmed by RNAi knockdown and in three independent alleles, whose lesions affect differing structural domains of the protein (Patel et al., 2008). Furthermore, these alleles represent both classes of daf-2 mutation: m596 being a member of the first class and affecting primarily dauer arrest; e1370 and e1391 being members of the second, exhibiting effects on dauer arrest and manifesting other, non-dauer defects, such as defects in oocyte development, gonad structure, and overall fertility, as well as significant L1 larval arrest in nutrient replete conditions (Gems et al., 1998; Patel et al., 2008). Differences in methodology could underlie my inability to replicate the results of Kenyon and colleagues (Pinkston-Gosse and Kenyon, 2007; Pinkston et al., 2006) since they examined only daf-2(e1370) mutants and confirmed cell death only with the DNA-binding dye SYTO-12. I examined multiple alleles of daf-2, including RNAi, and confirmed my counts directly, and with the DNA-binding dye acridine orange (see section 3.2). That differences in methodology may underlie my inability to replicate the findings of Kenyon and colleagues (Pinkston-Gosse and Kenyon, 2007; Pinkston et al., 2006) is supported by the inability of other studies, which used methods similar to my study, to replicate their findings. For instance, in addition to an anti-apoptotic function for daf-2, Kenyon and colleagues (Pinkston-Gosse and Kenyon, 2007; Pinkston et al., 2006) also reported a pro-apoptotic role for daf-16 in the germline. Two subsequent studies (Greiss et al., 2008; Quevedo et al., 2007) were unable to confirm this pro-apoptotic function for daf-16 and instead found a mild anti-apoptotic role for daf-16 in the germline. Because Kenyon and colleagues (Pinkston-Gosse and Kenyon, 2007; Pinkston et al., 2006) confirmed germ cell apoptosis in daf-2 and daf-16 mutants using only the DNA-binding dye SYTO-12, while later studies by Greiss
(2008) and Quevedo (2007) confirmed germ cell apoptosis both through direct microscopic examination and DNA binding dyes, Kenyon and colleagues may have missed important effects on cell death in *daf-2* and *daf-16* mutants. In addition, a recently published study supports my contention that *daf-2* is pro-apoptotic since Luo and colleagues (2010) found that *daf-2(e1370)* mutants are resistant to IR-induced germ cell apoptosis. Interestingly, Luo and colleagues (2010) used only SYTO-12 staining to label dying cells in their study. Combined, published studies agree with my contention that *daf-2* is required for DNA damage-induced germ cell apoptosis.

Previous studies of PI3K signalling have been restricted to cell culture and more recent attempts to delineate the function of the pathway in whole tissues have demonstrated that its linearity is not always preserved. For instance, studies of how deregulated Akt signalling controls development of the mouse brain demonstrated that the effects of PDK1 on Akt can vary between neurons and glia (Chalhoub et al., 2009). In addition, recent reports have shown that the output of PI3K signalling in many cancers is often not Akt activation. Many kinases in addition to Akt rely upon PDK1 phosphorylation to attain full activation; as it does on Akt, this phosphorylation permits proper folding of the active site in p70 ribosomal protein S6 kinase (Williams et al., 2000) and protein kinase C (Newton, 2003). In the case of cancers containing activated PI3K, PDK1 targets Serum and Glucocorticoid-Inducible Kinase 3 (SGK3) to drive Akt-independent proliferation (Vasudevan et al., 2009). SGKs are close cousins of Akt that can function to regulate dauer arrest and lifespan as part of an AKT-1/AKT-2/SGK tripartite complex in *C. elegans* (Hertweck et al., 2004), although the sole worm SGK homologue SGK-1 (Manning, 2005) does not regulate damage-induced germ cell apoptosis (Celia Quevedo, unpublished observations). In addition, the wiring of the PI3K pathway has been shown to change, albeit not to the degree that I observe, in other cancer cells such that the phosphorylation profile of Akt targets differs markedly across cell type and cancer (Ericson et al., 2009). Some of these effects require Akt and others do not. All of these data point to the ability to modify the organization of signalling pathways in both health and disease (Grueneberg et al., 2008).
5.2 Multiple Parallel Signalling Pathways Promote Cell Death

The fact that \textit{pdk-1} can promote damage-induced apoptosis without altering the transcriptional function of CEP-1, yet still require the \textit{cep-1} locus strongly suggests that signalling from multiple pathways is required in parallel to promote cell death in the \textit{C. elegans} germ line; this represents the first direct evidence of such a situation (Figure 3.6). Previously, other studies had identified \textit{cep-1}-independent regulators, but it was unclear how important these pathways were to death signalling. For example, \textit{lin-35}, a homologue of the mammalian pocket protein Rb (Lu and Horvitz, 1998), was found to be required for damage-induced germ cell apoptosis independently of \textit{cep-1} (Schertel and Conradt, 2007). The ability of \textit{lin-35} to promote cell death was accomplished through repression of \textit{ced-9}, although the mechanistic details of this were not elucidated. Another recent study identified a key role for the gene \textit{kri-1} in promoting damage-induced apoptosis upstream of \textit{ced-9} as well (Ito et al., 2010). In this case, however, \textit{kri-1} did not regulate the expression of CED-9. Therefore, while \textit{lin-35} and \textit{kri-1} appear to function separately as \textit{cep-1}-independent pro-apoptotic regulators, how important their respective roles are in relation to \textit{cep-1} was unclear. My work has demonstrated that parallel signalling from \textit{pdk-1} is just as important for germ cell death as CEP-1-dependent \textit{egl-1} induction. This argues that multiple pathways must be competent simultaneously for damage-induced apoptosis to occur and forces us to re-evaluate the linear assumptions that we hold about \textit{cep-1}-dependent death in the germline (Derry et al., 2001; Schumacher et al., 2001), and by extension, death elicited by p53 in mammals.

In addition to their parallel signalling independent of \textit{cep-1}, \textit{lin-35} and \textit{kri-1} share another commonality: they regulate the cell death machinery at least partially (\textit{lin-35}) or completely (\textit{kri-1}) from outside the germline itself. \textit{lin-35} can provide pro-death signals from the gonadal sheath (Schertel and Conradt, 2007), a single-cell layer of somatic cells that surrounds the germline (Kimble and Hirsh, 1979), and it is possible that \textit{kri-1} could signal to damaged germ cells from the same location (Ito et al., 2010). \textit{daf-2}, which shares an epistatic position with \textit{lin-35} and \textit{kri-1} upstream of \textit{ced-9}, can regulate aging, and possibly dauer arrest, solely from the somatic nervous system (Apfeld and Kenyon, 1998; Wolkow et al., 2000). It is therefore possible that
*daf-2* could regulate apoptosis non-autonomously and that novel pathways exist to relay somatic death signals to germline CED-9 (Figure 5.1).

*pdk-1* differs from *daf-2, lin-35* and *kri-1* in that it appears to function independently of *ced-9* (Figure 3.4E). The fact that a null mutation in *ced-4* can suppress the sensitivity of *pdk-1*(gf) germ cells to DNA damage suggests that *pdk-1* functions upstream of *ced-4* to control apoptosis (Figure 3.4F). Another recently identified *cep-1*-independent regulator, *sir-2.1* (*sir* – yeast SIRtuin related), also has been suggested to regulate CED-4 (Greiss et al., 2008). Purportedly, *sir-2.1* can control the localization of CED-4 during apoptosis and the absence of *sir-2.1* impedes proper CED-4 condensation around the germ cell nucleus during death. The translocation of CED-4 from mitochondria to the nuclear membrane has been described as a key step in developmental apoptosis (Chen et al., 2000). The reported movement of CED-4 in the germline in response to DNA damage is far less obvious, instead appearing as the coalescence of CED-4 already on the nuclear membrane into distinctive puncta (Greiss et al., 2008). This could represent the localized oligomerization of CED-4 from inactive dimers to the octameric structure that activates CED-3 (Qi et al., 2010). Despite this conjecture, the significance of puncta formation to the execution of apoptosis is still not known since the puncta that we occasionally observed in irradiated germ cells were not reproducible (Figure 3.5) and since CED-4 does not appear to change localization in response to IR or in actively dying germ cells (Figure 3.5). The fact that I do not observe any changes in CED-4 levels or localization after DNA damage in the wild-type argues against an important role for CED-4 puncta formation in the execution of apoptosis and instead suggests that *pdk-1* regulates other aspects of CED-4 function, such as interaction with CED-3, or post-translational modifications that facilitate its function (Figure 5.1).
AKT-1 prevents DNA damage-dependent activation of CEP-1 by interfering with the functions of the ATR homologue ATL-1. AKT-1 probably targets multiple aspects of ATL-1 function (dashed lines), including recruitment of ATL-1 to sites of DNA damage and its subsequent activation by MUS-101 and the 9-1-1 complex. An unknown pathway controls this germline-intrinsic function of AKT-1 (represented by question mark). DAF-2 and PDK-1 control apoptosis independently of AKT-1: DAF-2, like LIN-35 (Schertel and Conradt, 2007) and KRI-1 (Ito et al., 2010), functions through CED-9; PDK-1 signals through CED-4. The site(s) of action for DAF-2 and PDK-1 are not known. Hypothetically, they could reside outside of the germline (dashed lines). SIR-2.1 is reported to control the subcellular localization of CED-4 from inside damaged germ cells (Greiss et al., 2008).

Multiple parallel signalling events being required for damage-induced apoptosis leads one to question whether all function constantly, or whether there are some pathways that can be induced at the correct moment to execute apoptosis. With the current information, it seems likely that...
CEP-1 represents one candidate to perform the latter function. *egl-1* is expressed at low levels in undamaged germ cells and this expression rises robustly after DNA damage (Hofmann et al., 2002). The dynamic nature of CEP-1 phosphorylation (Quevedo et al., 2007) and inferences from the regulation of mammalian p53 proteins (Lavin and Gueven, 2006) suggest that activation of CEP-1 by upstream kinases such as ATL-1 would allow the rapid input of a rate-limiting signal into a “primed” system of *daf-2, pdk-1, lin-35, sir-2.1*, and *kri-1*. The implication is then that CEP-1 “tips the balance” of the system to favour cell death. Uncovering the mechanisms by which the above *cep-1*-independent regulators control apoptosis will provide a useful test of this model of germ cell death.

5.3 A Novel Regulatory Mechanism for Akt

My demonstration of *pdk-1*-independent regulation of *akt-1* directly challenges the notion that PDK1 is always the key activator of Akt proteins (Alessi et al., 1996a; Burgering and Coffer, 1995; Datta et al., 1996; Franke et al., 1995; Kohn et al., 1995). Furthermore, the potential for such a situation, albeit in different contexts, was hinted at in previous studies wherein *C. elegans* AKT proteins were found to have a strong role in conferring sensitivity to bacterial pathogens, while *pdk-1* status had little effect (Evans et al., 2008). Thus, factors other than PDK-1 may regulate the *C. elegans* AKT proteins in response to infection. In dauer arrest, certain gf mutations in *akt-1* are able to bypass the constitutive dauer arrest of *age-1* null mutants in the absence of *pdk-1* (Gami et al., 2006). Because the suppressive effects of *akt-1(gf)* mutations in *age-1(0)* mutants regularly require *pdk-1* (Paradis et al., 1999), additional regulators may function to control *akt-1* in the absence of PI3K activity in the worm soma. Further support for this comes from mammals where heat shock and hyperosmolarity can activate Akt independently of PI3K in cultured cells (Konishi et al., 1996).

Analysis with phospho-specific antibodies supports these genetic observations and suggests that *pdk-1* status does not significantly affect T350 phosphorylation in AKT-1 (Figure 3.9). By phosphorylating T308 in mammalian Akt, PDK1 allows the activation loop to adopt an extended conformation that frees its steric obstruction of the active site (Yang et al., 2002a) (Figure 5.2).
This, coupled with S473 phosphorylation, allows the Akt active site to adopt its active conformation (Yang et al., 2002b). It is surprising then that changes in pdk-1 status have little affect on T350 phosphorylation in response to DNA damage, and that, apparently, AKT-1 can presumably function without T350 phosphorylation in daf-2(lf) mutants (Figure 3.9). Because re-organization of the activation loop into an extended conformation is an essential part of kinase activation (Nolen et al., 2004), a novel mechanism must facilitate its reorganization in AKT-1 in the absence of T350 phosphorylation in response to DNA damage. It is possible that another kinase could mediate T350 phosphorylation, although if one assumes that AKT-1 is still active in daf-2(lf) mutants, where T350 phosphorylation is significantly impaired, this would be hard to reconcile. The development of in vitro assays to measure AKT-1 kinase activity would provide important insight into this question.

Figure 5.2 Phosphorylation in the activation loop of kinases clears the active site for catalysis.

The amino- and carboxy-terminal lobes (N- and C-lobes, respectively) of the kinase domain are held in close proximity by a linker region; the active site lies in the cleft between these two lobes. In the inactive state, a portion of the C-lobe termed the activation loop (yellow) folds back in to the active site, disrupting enzyme-substrate interactions. In general, phosphorylation of Ser, Thr or Tyr residues within the activation loop causes it to adopt a conformation that extends away from the active site (Nolen et al., 2004). This frees the active site from steric hindrance and allows the extended activation loop to participate in substrate binding.
Alternatively, attention of an extended conformation in the activation of AKT-1 could be mediated through direct binding of another protein to portions of AKT-1, including the activation segment. Such a mechanism is elegantly illustrated by the activation of cyclin-dependent kinases (CDK) by their cognate binding partners, the cyclins. By binding on one side of the catalytic cleft, cyclins can directly interact with the activation loop of the CDK, drawing it into the extended form that facilitates kinase activation (Jeffrey et al., 1995). In most CDKs, this is followed by phosphorylation of a threonine in the activation loop that further extends it and ensures maximal kinase activation; however, two recent studies have demonstrated that a virally-encoded cyclin and a cyclin-like protein, p25, can fully activate CDK6 and CDK5, respectively, without phosphorylation of the activation loop. This is achieved by expanding the CDK-binding interface on the cyclin or cyclin-like protein such that the fully active conformation of the activation loop can be maintained without further post-translational modification (Schulze-Gahmen and Kim, 2002; Tarricone et al., 2001). The activation of the protein kinase LKB1/STK11 (serine/threonine protein kinase 11) also follows a similar, phosphorylation-independent approach to activation loop extension (Boudeau et al., 2004). In this case, two separate binding partners, STRAD (STE20-related adapter protein) and MO25 (mouse embryo scaffolding protein 25), bind on opposite sides of STK11 to mediate kinase activation. Specifically, MO25 makes direct contact with the activation loop, extending it out of the active site cleft and, in concert with the allosteric effects of STRAD binding on the opposite face of STK11, allows complete activation of STK11 without activation loop phosphorylation (Zeqiraj et al., 2009).

It is possible that DNA damage-dependent activation of AKT-1 proceeds via a similar binding partner-dependent mechanism. Perhaps priming phosphorylation on S517 facilitates activation loop extension by an unidentified AKT-1 binding partner (Figure 5.3A). Indeed, phosphorylation at S473 has been shown to prime mammalian Akt for activation at T308 (Scheid et al., 2002). If such an overall approach to kinase activation is conserved, it will be important to test this by searching for novel AKT-1 binding partners by affinity purification and mass spectrometry. Finally, to fully exclude a role for T350 and S517 in damage-dependent AKT-1
regulation, it will be important to assess the ability of T350A and S517A mutants to rescue the hypersensitivity to damage-induced germ cell apoptosis in *akt-1(ok525)* mutants. This will require the generation of transgenic worms that express *akt-1* in the germline (see Figure 3.10).

Alternatively, it could be that AKT-1 is regulated by phosphorylation, albeit at sites other than T350 and S517 (Figure 5.3B). In the co-translational maturation of mammalian Akt, mTORC2 phosphorylates T450 in a growth-factor independent manner (Facchinetti et al., 2008; Ikenoue et al., 2008). This renders Akt responsive to further growth factor-derived signals (Bellacosa et al., 1998). T450 is conserved as T492 in AKT-1 and although the growth factor-independence of this phosphorylation site strongly suggests that it is facilitative for kinase activity rather than regulatory, its conservation does raise the possibility that AKT-1 could be regulated, at least in part, by phosphorylation at other unidentified residues. Perhaps phosphorylation at some of these potential sites, which are scattered throughout the PH and kinase domains of AKT-1, could contribute to regulation by a binding partner-co-phosphorylation mechanism. Future studies of AKT-1 phosphorylation sites by mass spectrometry will support or refute this hypothesis.
Figure 5.3 An activation loop binding partner-dependent mechanism for AKT-1 activation.

**A)** A DNA damage-dependent activation partner of AKT-1 (purple) binds the activation loop (yellow) and facilitates kinase activation in the absence of T350 phosphorylation (T350 is located within the activation loop). It is possible that phosphorylation at S517 plays a role in priming AKT-1 activation in this model. **B)** It is also feasible that AKT-1 could be activated by phosphorylation at single or multiple unidentified residues scattered throughout the PH and kinase domains. This model does not exclude the participation of an activation loop binding partner that co-operates with phosphorylation at novel sites to drive DNA damage-dependent AKT-1 activation.
Overall, with the data that I have generated, I hypothesize that DNA damage-dependent AKT-1 activity is regulated by a combination of S517 phosphorylation-based priming and a binding partner that maintains the activation loop in an extended conformation without T350 modification. On the contrary, phosphorylation of T350 seems to be quite important in the control of AKT-1 in the soma, although whether this is mediated by PDK-1 is a matter of contention. Together, this could reflect fragmentation of the PI3K pathway to mediate unique outputs from single pathway components - unique activation methods could be coupled to differential phosphorylation.

Figure 5.4 Hypothetical functional output switching by stimulus-specific phosphorylation of AKT-1.

(Left) Phosphorylation on T350 in the activation loop drives AKT-1 to inhibit DAF-16 and avoid dauer arrest in the fed state. (Right) In response to DNA damage, S517 phosphorylation may prime AKT-1 so that it can in turn bind an unidentified partner (purple) that extends the activation loop, driving maximal catalytic activity. This configuration musters AKT-1 to dampen ATL-1-dependent pro-apoptotic signalling in the germline.
5.4 ATL-1 as a Downstream Target of AKT-1

ATL-1 represents a beguiling target for AKT-1. Complete loss of ATR in mammals (Brown and Baltimore, 2000; Brown and Baltimore, 2003) and ATL-1 in C. elegans (Garcia-Muse and Boulton, 2005) leads to aberrant chromosome segregation and mitotic catastrophe after only a few cell cycles. Only through maternal rescue am I able to generate viable atl-1(0) animals. Therefore, maternal atl-1 must be sufficient to allow the cell divisions necessary to generate somatic lineages in the worm. After this, mitosis in the distal region of the germline is disrupted in atl-1(0) mutants such that chromosomal aberrations become apparent (Garcia-Muse and Boulton, 2005). This has an important impact on fertility since although wild-type numbers of eggs are laid, none of them are viable. It seems likely that AKT-1 reaches a compromise by dampening, but not completely inhibiting ATL-1 activity in response to DNA damage. This could be accomplished by globally regulating ATL-1 kinase activity, regulating this activity only in certain areas of the germline (i.e. only in pachytene cells), or preventing the proper localization of ATL-1 to sites of DNA damage only in cells likely to die. Such an ATR-dampening approach is supported by recent studies in mammalian cells that found a role for estrogen-induced PI3K-Akt signalling in the dampening of UV-induced ATR function (Pedram et al., 2009). By interfering with the interaction of TopBP1 with ATR-ATRIP, Akt was able to reduce signalling through ATR-Chk1 and bypass the G2/M checkpoint without killing cells outright. This was proposed as an important way for breast, and other estrogen receptor-positive cancers to resist therapy (Pedram et al., 2009).

It is unfortunate that I was unable to obtain specific germline staining with my α-AKT-1 antibodies as this would allow me to distinguish where in the germline AKT-1 is likely to act, and whether ATL-1 also functions at these locations. In response to DNA damage, ATL-1 is recruited to sites of strand breaks and forms discernable puncta in mitotic and meiotic germ cells (Garcia-Muse and Boulton, 2005). It will be interesting to examine whether akt-1 status affects this recruitment process in all eligible germ cells (i.e. globally), or only in specific areas. If the latter is true, important clues to the site of action of AKT-1 in the germline would be provided. At the same time, it will be important to understand if ATL-1 kinase activity is altered in akt-1 mutant worms. Experiments are currently underway to examine the activity of ATL-1 in
response to DNA damage using the phosphorylation of CHK-1 as an output. This is an oft-used assay of ATR kinase activity (Lupardus et al., 2002) that has proven robust in C. elegans too (Kim et al., 2007; Lee et al., 2010). Because both total and location-specific ATL-1 activity can be monitored by Western blotting and whole mount staining of dissected germlines with phospho- and total-CHK-1 antibodies (Kim et al., 2007; Lee et al., 2010) this approach should allow the differentiation of global versus local effects of AKT-1. In totality, my data support a model of AKT-1 function that features dual inputs to ATL-1, one from DNA damage sensors such as hpr-9, mrt-2, hus-1, and clk-2 and the other from AKT-1 itself (Figure 5.5). Genetic evidence indicates a lag in the appearance of akt-1(RNAi)-induced hypersensitivity to IR when compared to wild-type (Figure 4.4B). This six hour delay in akt-1(RNAi) may be consistent with an IR-dependent regulatory mechanism that translates initial flux through the apoptotic pathway into a measured AKT-1 response.

![Figure 5.5 A model for the DNA damage-dependent regulation of AKT-1.](image)

The severity or amount of DNA damage, perhaps sensed by 9-1-1 loading/occupancy, is interpreted by an upstream pathway (question mark) that induces appropriate AKT-1 activity.

### 5.5 Implications for Cancer

If this hypothesized method of IR-specific Akt regulation is conserved in mammals, it could represent a powerful way to selectively combat the survival advantage of Akt-dependent tumours. Generally, Akt activity in cancers is correlated strongly with its phosphorylation (Liu
et al., 2009), but very few studies have examined Akt function beyond its phosphorylation state on Western blots and it could be that phosphorylation at T308 is not important for the response of tumours to IR. Although I have not conclusively shown T350/T308 to be dispensible for AKT-1 activity, it is possible that AKT-1 may function independently of T350/308 phosphorylation in response to DNA damage in *C. elegans*. If this IR-specific mechanism of Akt regulation is conserved in humans, and utilized by cancer cells for their survival, it could be exploited to specifically target certain tumours for therapy. The idea that IR-specific pathway controlling Akt can emerge in cancer is supported by a recent study. Shimura and colleagues (2010) mimicked fractionated radiotherapy of tumours (multiple low doses of IR distributed across a long time interval) and observed the development evolution of stable radioresistance in liver (HepG2) and cervical cancer (HeLa) cell lines. This radioresistance of fractionally-irradiated cells resulted from cyclin D over-expression that drove unchecked cell cycling and generated persistent DNA damage. This recurrent DNA damage activated DNA-PKcs and thence Akt, amplifying cyclin D levels by inhibition of GSK3β (Alt et al., 2000). The DNA-PKcs-Akt-GSK3β-cyclin D positive feedback loop discovered in this study, coupled with increased capacity for DNA repair in radioresistant cells, allowed retention of resistance after long breaks in the therapeutic regime and also permitted increased proliferation in the face of renewed therapy. Although Shimura and colleagues (2010) did not define the contribution of T308 phosphorylation to this Akt regulatory pathway, their data does suggest that cancers can develop resistance to IR through increased Akt function. Even though I have not demonstrated that the novel AKT-1 regulatory pathway that I report here is conserved in mammals, use of a similar mechanism in cancer cells might open avenues to the specific treatment of therapy-resistant tumours.
6 Future Directions

6.1 Regulation of DNA Damage-induced Germ Cell Apoptosis by \textit{daf-2}/InsR and \textit{pdk-1}/PDK1

Although epistatic analysis has suggested that \textit{daf-2} acts upstream of \textit{ced-9}/inhibits \textit{ced-9}, how DAF-2 inhibits CED-9 to promote apoptosis is unknown. Previous work has uncovered a role for the transcriptional repression of \textit{ced-9} by \textit{lin-35} to promote germ cell death (Schertel and Conradt, 2007). It is possible that \textit{daf-2} could perform a similar function, although the mechanism of \textit{ced-9} transcriptional regulation is likely to be indirect: both \textit{daf-2}, a receptor tyrosine kinase, and \textit{lin-35}, encoding the \textit{C. elegans} homologue of the Rb tumour suppressor, are not transcription factors themselves; thus they probably function through an intermediary. By signalling through an intracellular pathway, or by binding to and sequestering factors necessary for \textit{ced-9} expression (Schertel and Conradt, 2007), respectively, \textit{daf-2} and \textit{lin-35} could control \textit{ced-9} expression. In the context of lifespan regulation and dauer arrest, \textit{daf-2} regulates the transcriptional function of the Forkhead transcription factor DAF-16 (Lin et al., 1997; (Ogg et al., 1997). \textit{daf-2} can function at least partially through \textit{daf-16} in the regulation of germ cell apoptosis too since a null mutation in \textit{daf-16} partially reverts the resistance of \textit{daf-2(e1370)} mutants to IR (Figure 6.1A). As a transcription factor DAF-16 could in turn regulate \textit{ced-9} expression although \textit{ced-9} has not been identified as a \textit{daf-16}-regulated gene (Pinkston-Gosse and Kenyon, 2007). Therefore, the mechanism by which \textit{daf-2} regulates damage-induced germ cell apoptosis independently of \textit{pdk-1} and \textit{akt-1} will require future studies. These could include examinations of the expression levels and localization of CED-9 as well as searches for potential post-translational modifications on CED-9, especially since DAF-2 presumably possesses tyrosine kinase activity (Kimura et al., 1997).

Alternatively, my data are consistent with \textit{daf-2} regulating apoptosis at the level of \textit{egl-1}.

Indeed, analysis of damage-dependent CEP-1 transcriptional function demonstrates that \textit{egl-1} is induced to greater than wild-type levels in \textit{daf-2} mutants (Figure 3.4B). While this could reflect the ability of \textit{daf-2} to negatively regulate CEP-1, it is also consistent with \textit{daf-2} regulating the stability of the \textit{egl-1} transcript. This could determine the expression levels of EGL-1 and it
would be interesting to examine this in *daf-2* mutants given that transcriptional regulation is one of the main mechanisms controlling the functions of BH3-only proteins (Danial, 2009). It is also possible that DAF-2, as a kinase, controls the subcellular localization or function of EGL-1 by phosphorylation. One of the first apoptotic targets of Akt identified was the pro-apoptotic BH3-only protein BAD (BAD - Bcl2 antagonist of cell death) (Datta et al., 1997). Akt-dependent phosphorylation of BAD at Ser136 causes it to bind to 14-3-3 proteins and become sequestered from its normal target, the anti-apoptotic Bcl2 (CED-9 in *C. elegans*) (Datta et al., 2000). While AKT-1 regulates apoptosis through ATL-1 (this work), and EGL-1 does not contain any predicted Akt consensus phosphorylation sites (data not shown), it is possible that DAF-2 indirectly controls the phosphorylation state and function of EGL-1 through a kinase cascade. If true, loss of 14-3-3 proteins [there are 3 predicted 14-3-3 proteins in *C. elegans* (Plowman et al., 1999)] should be able to revert the apoptosis defects in *daf-2* mutants. Unfortunately, many of these proposed experiments are hampered by the technical limitations imposed by EGL-1 – it is difficult to express in bacteria and human cells and, when expressed, is not particularly immunogenic. Thus, antiseras against EGL-1 have proved exceedingly difficult to raise (Anton Gartner, Barbara Conradt, personal communications). EGL-1-specific antiseras and/or germline-expressing transgenics will be required to further characterize EGL-1 protein levels or its localization in *daf-2* mutants. It is also possible that the total loss of AKT-1 T350 and S517 phosphorylation in *daf-2* mutants (Figure 3.9A) renders AKT-1 non-functional, lifting inhibition of CEP-1 (Figure 3.4A) and leading to excessive *egl-1* induction in response to DNA damage (Figure 3.4B). Optimized biochemical assays that can quantify AKT-1 activity in *daf-2* mutants are required to either confirm or refute such a model.
Figure 6.1 Loss of daf-16 only partially reverts the resistance to apoptosis of daf-2 mutants.

A) L4 stage worms were treated with IR and germ cell apoptosis was quantified after 24 hours at 25°C. daf-2(lf) = daf-2(e1370), daf-16(0) = daf-16(mgDf47). Error bars are as in Figure 3.2. B) L4 stage worms (raised from the L1 stage on cep-1 or control RNAi) were treated with IR, incubated for 24 hours at 25°C, and total RNA was then isolated. egl-1 transcript levels were quantified by Real Time PCR, using tbl-1 as an internal control. Error bars represent the SEM from at least three independent experiments.

Because pdk-1 does not appear to regulate the protein levels or the subcellular localization of CED-4 (Figure 3.5), it will be interesting to examine whether it might control CED-4 post-translational modifications, particularly phosphorylation. This is not without precedent in mammals, where the CED-4 homologue APAF1 is phosphorylated by protein kinase A (PKA) to temper its apoptosis-inducing activity (Martin et al., 2005). Since PKA can be activated by IR in human cancer cells (Sanli et al., 2010), and since PDK1 serves as a key activator of protein kinase C and Akt, both close cousins of PKA (Newton, 2003), it is possible that PDK-1 could control CED-4 activity through either of the PKA-like kinases in C. elegans, KIN-1 or F47F2.1 (KIN – protein KINase). However, probably the best method to identify targets of daf-2 and pdk-1 will be an unbiased forward genetic suppressor screen. Mutagenizing daf-2(lf) or pdk-1(lf) mutants and directly screening for rescue of damage-induced germ cell apoptosis using acridine orange staining would best exploit the powers of C. elegans as a model organism.
Another key question to address is from where \textit{daf-2} and \textit{pdk-1} regulate damage-induced apoptosis. The recently described \textit{cep-1}-independent cell death regulators \textit{lin-35} and \textit{kri-1} both function from outside of the germline proper: \textit{lin-35} activity being provided by the somatic gonadal cells (Schertel and Conradt, 2007); and \textit{kri-1} activity being provided exclusively from an unidentified somatic source (Ito et al., 2010). The ability of \textit{daf-2} to regulate the lifespan of all cells in the worm from only a few neurons in the head (Wolkow et al., 2000) and to control dauer arrest of the whole organism from a small subset of tissues including neural and gut (Apfeld and Kenyon, 1998), raises the possibility that germ cell apoptosis could be regulated non-autonomously by \textit{daf-2} as well. This is supported by the recent observation that ASJ neurons in the head of the worm can regulate the secretion of a diffusible factor that controls damage-induced germ cell death (Sendoeel et al., 2010). Also, ongoing experiments show that \textit{daf-2} is required in multiple somatic tissues and the germline to regulate apoptosis (Shu Ito, personal communication); perhaps these somatic tissues could include neurons in the head. By further exploring the expression requirements for \textit{daf-2} and \textit{pdk-1} using tissue-specific expression constructs, the site of action of these two regulators can be defined.

6.2 Activation and Control of AKT-1/Akt in Response to DNA Damage

Separation of AKT-1 from canonical PI3K regulatory elements poses a pressing problem for kinase regulation - Akt proteins generally require phosphorylation to maintain their active state (Alessi et al., 1996a). If PDK-1 does not modulate this phosphorylation in \textit{C. elegans}, then it follows that another kinase might. Therefore, it will be very informative to screen the \textit{C. elegans} kinome [~438 predicted kinases (Manning, 2005)] by RNAi to identify candidates that could function as DNA damage-specific activators of AKT-1. It is also important to consider that DNA damage-dependent regulation of AKT-1 could proceed by a unique mechanism that does not involve canonical phosphorylation sites. Examination of the phospho-status of the T350 and S517 sites in AKT-1 after IR indicated that S517 may represent an activation target on AKT-1 (Figure 3.9B). Therefore, it will be interesting to examine the ability of putative S517 kinase such as TORC2 to regulate AKT-1 in a damage-dependent manner using \textit{rict-1} (RNAi). My previous attempts to examine TORC2 function in germ cell apoptosis were hampered by the
severe germline proliferation defects observed in \textit{rict-1} null mutants. Perhaps \textit{rict-1} RNAi will allow me to circumvent these limitations and to assess whether loss of TORC2 activity reverts the damage-induced increases in AKT-1 S517 phosphorylation.

An unexpected finding of this work was that removal of \textit{pdk-1} resulted in doubling and even tripling of S517 phosphorylation both in the absence and presence of damage (Figure 3.9). This strongly suggests that a loop exists whereby loss of \textit{pdk-1} de-represses S517 kinase activity. As discussed in the results (section 3.9), this is unlikely to result from de-phosphorylation of IST-1, the \textit{C. elegans IRS1} homologue, but rather is likely to proceed by a unique mechanism. Because loss of \textit{daf-2} does not generate the same effect, it seems that this loop is restricted to \textit{pdk-1} and not the affected generally by the PI3K pathway. As a first step in the characterization of this new \textit{daf-2}-independent loop, it will be interesting to examine whether simultaneous loss of \textit{daf-2} will suppress activation of S517 phosphorylation in \textit{pdk-1(lf)} mutants. The homologous S473 phosphorylation feedback loop described in \textit{Drosophila} (Radimerski et al., 2002; Yang et al., 2006) requires the presence of dInsR for feedback upregulation of S473 phosphorylation in the absence of TORC1 activity (Kockel et al., 2010), so these experiments will allow the differentiation between two models: one in which feedback regulation of S517 phosphorylation results from a \textit{daf-2}-dependent kinase; or the other, in which \textit{daf-2} and \textit{pdk-1} have genetically separable effects on S517. Based on the genetic independence of these two genes from each other in the regulation of germ cell apoptosis, the latter model seems most likely at present. The simultaneous observation that loss of \textit{pdk-1} does not compromise AKT-1 phosphorylation while loss of \textit{daf-2} completely abrogates this process in unirradiated worms (Figure 3.9) directly conflicts with current models of dauer signalling as a linear phosphorylation cascade (Gami et al., 2006; Hertweck et al., 2004; Paradis et al., 1999). Although outside the scope of this thesis, future examination of the ability of PDK-1 to directly phosphorylate AKT-1 in dauer signalling will be important to clarify whether PDK-1 kinase activity \textit{per se} is required to regulate AKT-1 in this context. Perhaps, like STRAD and MO25 in the case of STK11, PDK-1 only needs to bind to AKT-1 to facilitate its function in dauer arrest. Furthermore, the identity of the \textit{daf-2}-dependent, \textit{pdk-1}-independent kinase that phosphorylates AKT-1 will represent an interesting avenue of investigation in future studies of dauer arrest.
An existing limitation to my studies of AKT-1 phosphorylation is the current inability to distinguish compartment-specific phosphorylation of AKT-1. Because the current antisera immunoprecipitate total AKT-1 from whole worms, it may be that certain tissue-specific effects on T350 and S517 phosphorylation are obscured. For example, I am currently unable to glean how much germline versus somatic AKT-1 is present in each of these immunoprecipitations. By examining the expression and phosphorylation of AKT-1 in germline defective mutants of *C. elegans*, I could begin to address these limitations.

It will also be important to investigate other mechanisms that could regulate AKT-1, such as the existence of a unique activation loop binding partner and/or alternative phosphorylation sites to T350 and S517. Fortunately, my antibodies that can immunoprecipitate AKT-1 from whole worms should allow the examination of potential binding partners and phosphorylation sites by mass spectrometry concurrently; other approaches to search for novel activation loop binding partners could include yeast two-hybrid screens using the kinase domain of AKT-1 as bait. Should additional phosphorylation sites be identified, it will be essential to examine their in vivo validity by mutating the relevant residue in transgenes and assessing whether this can rescue the sensitivity to damage-induced germ cell apoptosis caused by *akt-1* null mutations. In the course of these proposed experiments, further experimental evidence for the role of T350 and S517 phosphorylation in AKT-1 activation can be sought using the relevant site-directed transgenics.

Another important question is whether the kinase activity of AKT-1 is required to regulate apoptosis *per se*. Because I was unable to address this directly in *in vitro* kinase assays, it will be important to further develop purification conditions so that active AKT-1 can be obtained for use in biochemical assays. This will allow me to test whether AKT-1 is active in the absence of T350 and S517 phosphorylation in *daf-2(lf)* mutants. A complementary approach will be to also introduce kinase-dead transgenes into *akt-1(0)* mutants and assess the ability of these transgenes to restore wild-type levels of apoptosis. Should AKT-1 kinase activity not be required to
regulate apoptosis, this would require a fundamental re-evaluation of our conceptions about Akt function *in vivo*.

### 6.3 Are The Observed Inversions and Bifurcations in *C. elegans* Phosphatidylinositol 3-kinase Signalling Conserved in Mammals?

The existence of a DNA damage-specific conformation of the PI3K pathway in *C. elegans* argues that a single signalling pathway is used differently in different situations. This has implications for how we think about the signalling process. The PI3K pathway has been shown to govern a repertoire of cellular responses that is evolutionarily conserved (Manning and Cantley, 2007). *C. elegans* uses single homologues of PI3K pathway components to simultaneously control metabolism, aging, developmental decisions, and DNA damage-induced germ cell apoptosis. In certain situations (dauer arrest), the pathway functions linearly (Paradis et al., 1999; Paradis and Ruvkun, 1998) while in others (DNA damage-induced germ cell apoptosis), it is fragmented (this work). This suggests that the worm PI3K pathway is much more functionally flexible than previously thought. Therefore, the worm represents a strong model in which to examine the structural alteration of signalling pathways as a means to regulate distinct processes in the whole organism (Figure 6.2).
Figure 6.2 Stimulus- and output-specific selection of phosphatidylinositol 3-kinase signalling proteins may represent a conserved mechanism to generate functional diversity.

In different tissue or functional contexts, differing combinations of PI3K components can be selected to generate unique signalling outcomes. The linearity of PI3K signalling in many mammalian processes (growth, metabolism, survival in response to growth factors) is maintained. In the context of cancer, shuffling of downstream targets can generate unique outputs of PI3K (Vasudevan et al., 2009). *C. elegans* displays greater flexibility in PI3K pathway.
organization, probably a result of little functional redundancy. While developmental arrest decisions (Dauer) rely upon an intact, linear, PI3K pathway (Paradis et al., 1999; Paradis and Ruvkun, 1998), proper lifespan mostly bypasses AKT signalling (Evans et al., 2008), although some contributions of akt-1 through daf-16d/f may be present (Kwon et al., 2010). Pathway structure in response to infection (Evans et al., 2008) and DNA damage (this work) is significantly dispersed, even containing functional inversions that oppose one another (DNA damage). The key question then becomes: how are individual components in the fragmented conformations of the pathway regulated, and what are their targets?

Because much of the analysis of signalling in mammals has been undertaken in cultured cells, it will be very interesting to test whether the alterations in PI3K signalling in C. elegans will re-emerge in whole mammals or whether acquired functional redundancy has rendered this approach obsolescent. In particular, the discovery of parallel signalling from cep-1 and pdk-1 to promote cell death suggests that additional pathways will be required for p53-dependent death in mammals. In the context of cancer, this might explain why neoplastic cells mutate many other genes in addition to p53 to prevent cell death. Comprehensive studies of single genes and their contribution to the ability of cells to die in response to DNA damage may help to uncover the complex pro-apoptotic push that must be undertaken by moribund cells. Perhaps such a scenario provides a failsafe mechanism that can only be overcome by overwhelming ascent from multiple independent pathways.

If AKT-1 is indeed regulated by an IR-specific phosphorylation pathway (see Figure 5.5) it will be imperative to determine if this is conserved. Using site-directed mutants in C. elegans and mammalian cells, it should be possible to specifically assess contributions of T308 and S473 homologues to Akt activation in multiple circumstances. Perhaps past experimentation simply missed these situation-specific regulatory changes because of blunt tools. If such studies do reveal the conservation of a PDK1-independent regulatory pathway for Akt, this will have important implications in the development of targeted therapeutics that can specifically combat Akt-dependent tumours.
6.4 How Does AKT-1/Akt Regulate ATL-1/ATR to Dampen Pro-apoptotic Signalling?

If \textit{akt-1} can dampen apoptotic signalling by disrupting \textit{atl-1}-dependent signalling to \textit{cep-1}, this should be reflected in CEP-1 phosphorylation status and consequently, transcriptional function. ATR can directly phosphorylate human p53 (Hall-Jackson et al., 1999; Lakin et al., 1999; Tibbetts et al., 1999), and one would expect that such a relationship is conserved in \textit{C. elegans}. Thus, changes in ATL-1 activity brought about by alterations in \textit{akt-1} should directly affect the phosphorylation state of CEP-1. Furthermore, the hyperphosphorylation of CEP-1 seen in the absence of \textit{akt-1} (Quevedo et al., 2007) should be reverted by simultaneous loss of \textit{atl-1}. By performing Western blot analysis of CEP-1 mobility shift following DNA damage (Quevedo et al., 2007), the integrity of an AKT-1-ATL-1-CEP-1 axis could be directly read out and these changes correlated to CEP-1 transcriptional function by quantification of \textit{egl-1} expression.

ATR proteins preserve as one of their main targets the CHK1 family of protein kinases (Lupardus et al., 2002). Since CHK1 is conserved in \textit{C. elegans}, it will be interesting to determine whether loss of \textit{chk-1} is able to suppress the excessive damage-induced germ cell apoptosis in \textit{akt-1(0)} mutants. Previous work has indicated a controversial role for \textit{chk-1} in promoting germ cell apoptosis (Kalogeropoulos et al., 2004; Stergiou et al., 2007). Such experiments should help to resolve this dilemma. The partial rescue of \textit{mus-101} knockdown by concurrent loss of \textit{akt-1} (Figure 4.7) is reminiscent of the partial rescue of apoptosis in \textit{hus-1} and \textit{mrt-2} mutants by \textit{akt-1(0)} (Quevedo et al., 2007). Biochemical data demonstrates that ATR can be independently activated by TopBP1 (Kumagai et al., 2006) and the 9-1-1 complex (Majka et al., 2006b), but that complete activation requires both regulators \textit{in vitro} (Delacroix et al., 2007; Lee et al., 2007). By constructing \textit{mus-101hus-1; akt-1} and \textit{mus-101; mrt-2; akt-1} triple mutants, one could assess whether such a model is possible \textit{in vivo}. At the same time, it would provide insight into the linearity of the \textit{atl-1} pathway in the \textit{C. elegans} germline.
References


Wormbase. release WS219.


